Universidade de Aveiro Departamento de Biologia
Ano 2019

Mayara Pereira Soares

TOXICIDADE DO BARRAGE®, UM PESTICIDA À BASE DE CIPERMETRINA, EM LARVAS DE CAMARÕES E PEIXE ZEBRA

TOXICITY OF THE CYPERMETHRIN-BASED PESTICIDE BARRAGE® TO LARVAE OF SHRIMP AND ZEBRAFISH

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia e Ecologia das Alterações Globais, realizada sob a orientação científica do Doutor Amadeu Mortágua Velho da Maia Soares, Professor Catedrático do Departamento de Biologia da Universidade de Aveiro e co-orientação da Doutora Paula Inês Borralho Domingues, Investigadora em pós-doutoramento do Departamento de Biologia da Universidade de Aveiro e Liliam de Arruda Hayd, Professora adjunta da Universidade Estadual de Mato Grosso do Sul-Brasil.

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Palavras-chave

Ecotoxicologia, camarões, peixe-zebra, Pantanal, parâmetros ambientais, pesticidas, formulações, pH, nitrito, toxicidade, biomarcadores comportamentais, biomarcadores histológicos.

Resumo

O Pantanal (Brasil) é uma das maiores áreas úmidas do mundo e abriga uma considerável diversidade de organismos aquáticos. A região é baseada em um ciclo hidrológico onde a cada ano os rios inundam áreas extensas e períodos de seca que retiram a água para os leitos dos rios, com importantes variações nas propriedades físico-químicas da água de rios e lagoas. Associado a isso tem sido observado um crescimento cada vez maior de atividades agrícolas e da pecuária que dependem fortemente de produtos quimicos (pesticidas) para garantir a produção na região. Os efeitos desses compostos no ambiente, principalmente nos corpos d'agua não são muito bem conhecidos. Assim avaliar os efeitos desses compostos para a fauna aquática tendo em consideração variação ambiental (a qual pode ser ainda exacerbada com as atuais mudanças do clima) torna-se pertinente. Assim, o objetivo deste trabalho foi avaliar os efeitos do Barrage®, um pesticida a base de cipermetrina amplamente utilizado na região, em espécies de camarão (uma endêmica do Pantanal) e no peixe-zebra. Numa primeira fase foram estudados os efeitos de variações de pH ou nitritos na toxicidade do Barrage® para larvas de *Macrobrachium pantanalense*, *Macrobrachium amazonicum* e *Danio rerio*. Numa segunda fase os efeitos histológicos do Barrage® foram avaliados em adultos de *M.pantanalense*. Finalmente larvas de *Palemons varians* foram testadas como modelo para avaliação comportamental (locomoção) e a toxicidade comportamental do Barrage® testada nesta espécie e em larvas de *D. rerio*. Os resultados comprovam que o pH e nitrito modificaram a toxicidade do Barrage® nas espécies testadas, principalmente a nível subletal (crescimento e desenvolvimento larval). Os resultados sugerem que a letalidade pode não ser suficiente para prever corretamente os efeitos combinados de estressores e que a inclusão de espécies endémicas é fundamental para uma correta avaliação de risco ecológico em biomas sensíveis como o Pantanal. As larvas do camarão *P. varians* revelaram ser um bom modelo para avaliação da atividade locomotora e foram sensíveis aos efeitos do Barrage® confirmando a utilidade dos parâmetros comportamentais na detecção precoce de risco ecológico e indicando que o grupo dos camarões tem potencial como modelos em ecotoxicologia comportamental. A nível histológico, os resultados mostraram importantes alterações nas brânquias do camarão do Pantanal, incluindo lesões estruturais relevantes que podem afetar a função fisiológica destes organismos. Os resultados desta tese contribuem para uma melhor compreensão dos efeitos do Barrage® no desenvolvimento, histologia e comportamento de organismos não alvo, confirmando ainda a importância do estudo das variações ambientais na toxicidade deste composto. Estes dados são essenciais para chamar a atenção para a necessidade de conduzir estudos sistemáticos na região para previsão de riscos ecológicos do uso de pesticidas e estabelecimento de medidas de mitigação e proteção ambiental no Pantanal.

keywords

Ecotoxicology, shrimp, zebrafish, Pantanal, environmental parameters, pesticides, formulations, pH, nitrite, toxicity, behavioral biomarkers, histological biomarkers.

Abstract

The Pantanal (Brazil) is one of the largest wetlands in the world and is home to a considerable diversity of aquatic organisms. The region is based on a hydrological cycle where each year the rivers flood extensive areas and periods of drought return the water to the river beds, with important variations in the physicochemical properties of the water of rivers and lagoons. Associated with this it has been observed an increasing growth of agricultural and livestock activities that heavily depend on chemical products (pesticides) to ensure production in the region. The effects of these compounds on the environment, especially in water bodies, are not very well known. Thus, it is important to assess the effects of these compounds on aquatic fauna taking into consideration environmental variation (which can still be exacerbated in a climate change scenario). Therefore, the objective of this work was to evaluate the effects of Barrage®, a cypermethrin-based pesticide widely used in the region in shrimp species (one endemic to the Pantanal) and zebrafish model. In a first phase, effects of pH and nitrite concentrations variation in the toxicity of Barrage® were studied using larvae of *Macrobrachium pantanalense*, *Macrobrachium amazonicum* and *Danio rerio*. In a second phase, histological effects were studied in adults of *M. pantanalense*. Finally, larvae of *Palaemons varians* were tested as model for behavioral analysis (locomotion) and behavioral toxicity of Barrage® tested in this species and in *D. rerio* larvae. The results of this thesis confirm that pH and nitrite modify the toxicity of Barrage® to the tested species, mainly at sublethal level (larval growth and development). Results suggest that lethality may not be sufficient to correctly predict the combined effects of stressors and that inclusion of endemic species is crucial to the correct risk evaluation on sensitive biomas such as Pantanal. *P. varians* larvae revealed to be a good model for locomotor activity evaluation and were sensitive to the Barrage® effects confirming the usefulness of behavioral endpoints in the early detection of ecological risk and suggesting that shrimps as suitable models for behavioral ecotoxicology. At histological level, results show important changes in Pantanal shrimp gills, including relevant structural lesions that may affect the physiological function of these organisms. The results of this thesis contribute to a better understanding of the Barrage® effects in the development, behavior and histology of non-target organisms, also confirming the importance of the variation of environmental components in the toxicity of this compound. Data obtained are essential to raise awareness for the need of conducting systematic studies for accurately predicting ecological risks of the use of pesticides and establishing mitigation and environmental protection measures in Pantanal.

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Chapter 1

General Introduction

1. General Introduction

1.1 Contextualization

The Pantanal, classified by UNESCO as World Natural Heritage and Biosphere Reserve, is one of the largest wetlands in the world, providing multiple ecosystem services and playing an important role in the conservation of biodiversity (WWF, 2015). This fragile biome is based on a hydrological cycle where every year extreme rainfall causes rivers to flood extensive areas and then drought periods retreat water to river beds (Alho, 2008; Alpizar et al., 2011; Junk et al., 2006; Pott and Pott, 2004). Significant economic development has been observed in recent years in the region and one of the growing sectors is livestock and agriculture (Alpizar et al., 2011; Galdino et al., 2006; Ross and Sanches, 2006). These activities strongly rely on the use of chemicals (agrochemicals, pharmaceuticals) to assure productivity (Alpizar et al., 2011) but the undesirable effects of the use of these compounds in the environment, especially in the aquatic compartment have not been properly assessed.

Hydrological cycles verified in Pantanal correlate with important variation in water physic-chemical properties of rivers and ponds. This natural variation may be exacerbated in a context of climate changes where extreme events of rainfall and droughts are prone to occur. As well-known from scientific literature, environmental parameters may influence the toxicity of chemical compounds, and thus, in a scenario of increasing growth of agriculture and livestock sectors, is imperative to assess effects of agrochemical residues in water bodies taking into account environmental variables.

The present work aims to contribute to this challenge by studying the effects of the widely used cypermethrin based insecticide Barrage® on several shrimp species (one endemic from Pantanal) and on the model organism zebrafish. Were studied the influence of selected environmental parameters such as pH or nitrite levels on toxicity of Barrage® and its individual effect on endpoints as histology and behavior. The results of these studies are expected to provide important information that can be used to an accurate risk analysis of Barrage® use in Pantanal and ultimately contribute to a safe use of the compound in the region ensuring the environmental quality of this Biome.

1.2 Objectives

The objective of this thesis is to evaluate the effects of the cypermethrin-based insecticide Barrage® in aquatic species taking into account environmental variability here represented by variations in pH and nitrite concentrations. The species selected were the Pantanal endemic shrimp *Macrobrachium pantanalense*, the Amazon shrimp *Macrobrachium amazonicum* and the zebrafish (*Danio rerio*). Moreover, individual effects of Barrage® in very specific endpoints (histology and behavior) were studied in order to understand more subtle effects of the compound in non-target organisms. In order to reach the objectives, 3 specific aims were established:

1) To evaluate the influence of pH and nitrite variation on the toxicity of Barrage® using as model organisms the larvae of the endemic shrimp *Macrobrachium pantanalense*, larvae of the shrimp *M. amazonicum* (chapter 2) and embryos of zebrafish (*Danio rerio*) (chapter 3). The experiments described in these chapters were designed based on previous work from the author (Soares et al, 2017) where a characterization of the individual effects of Barrage® at acute and developmental level has been done in the 3 selected species.

2) To evaluate histological effects of Barrage® in gills of *Macrobrachium pantanalense* adults. Lethal toxicity of Barrage® to adult shrimps was characterized to and histological effects, already documented to other shrimp species evaluated.

3) To evaluate behavioral effects of Barrage® using of a shrimp species (*Palaemon varians*) and zebrafish. Since behavior has been recently considered a promising endpoint to assess effects of neuroactive drugs, methodologies used in zebrafish were adapted to larvae of *P. varians* (European species) and sensitivity of Barrage® tested to evaluate the potential of this endpoint and ultimately export these methodologies to be applied in the Brazilian species.

The work is divided into six chapters: Chapter 1 where a general introduction to different topics covered in the thesis is made; Chapters 2 to 5 present the results structured as scientific articles and Chapter 6 presents the conclusions obtained. The description of each chapter is summarized below:

Chapter 1: General introduction: contextualization of the work describing the Pantanal region, addressing the topic of climate change and its impact in Pantanal, the economic development of Pantanal, the use of agrochemicals, potential impacts on aquatic systems, ecotoxicological tools to address effects of contaminants and model species.

Chapter 2: Influence of pH and nitrites on the effects of Barrage® on two species of freshwater shrimp: In this study, the combined effect of Barrage® and pH or nitrite variations was studied in two shrimp species the endemic *Macrobrachium pantanalense* and the widespread *Macrobrachium amazonicum*. The endpoints evaluated were survival, growth and larval development.

Chapter 3: Influence of pH and nitrite on the effects of cypermethrin on zebrafish embryos: In this study, the combined effect of Barrage® and pH or nitrite variations was studied in zebrafish embryos. The endpoints evaluated were mortality, occurrence of cardiac edema, hatching, equilibrium, tail bending, yolk edema, yolk sac and lateral position.

Chapter 4: Histological changes induced by cypermethrin in gills of Pantanal endemic prawn *Macrobrachium pantanalense*: The toxicity of the Barrage® was studied by testing the Pantanal adult shrimp using several endpoints. Swimming impairment and gills histopathological changes were observed in shrimp exposed to Barrage®. This study shows the effects of Barrage® in ecological relevant endpoints in adult shrimps highlighting the need to monitor environmental levels of the compound and accurately assess the risks of Barrage® use.

Chapter 5: Behavioural effect of Barrage® in larvae of the shrimp *Palaemon varians*: This work assesses the suitability of the larvae of the shrimp *Palaemon varians* as model organisms to assess chemical effects on locomotor parameters. Zebrafish larvae behavioral methodologies were adapted to shrimp larvae and successfully used to evaluate behavioral effects of Barrage®. Behavioral endpoints are sensitive and ecological relevant and thus can be used in shrimps as a promising tool to accurately assess effects of contaminants.

Chapter 6: Discussion and concluding remarks: This chapter discusses the results obtained during the all thesis in a integrative way and summarizes the main highlights of the work.

2. Literature review

2.1 Pantanal

The Pantanal is located in the center of South America, located in the upper Paraguay basin and has an extension of 361666 km^2 distributed in Brazil (138183 km^2: 35.36% in the state of Mato Grosso and 64.64% in Mato Grosso do Sul), Bolivia and Paraguay (Junk and Nunes da Cunha, 2016). In Brazil Pantanal is divided in 11 subregions (Cáceres, Poconé, Barrão de Melgaço, Paraguay, Paiaguás, Nhecolândia, Abobral, Aquidauana, Miranda, Nabileque and Porto Murtinho) according to the drainage of the large rivers that form the hydrographic basin of the Paraguay River, an immense depressed area that drains the surrounding plateaus (Silva and Abdon, 1998) (Fig 1).

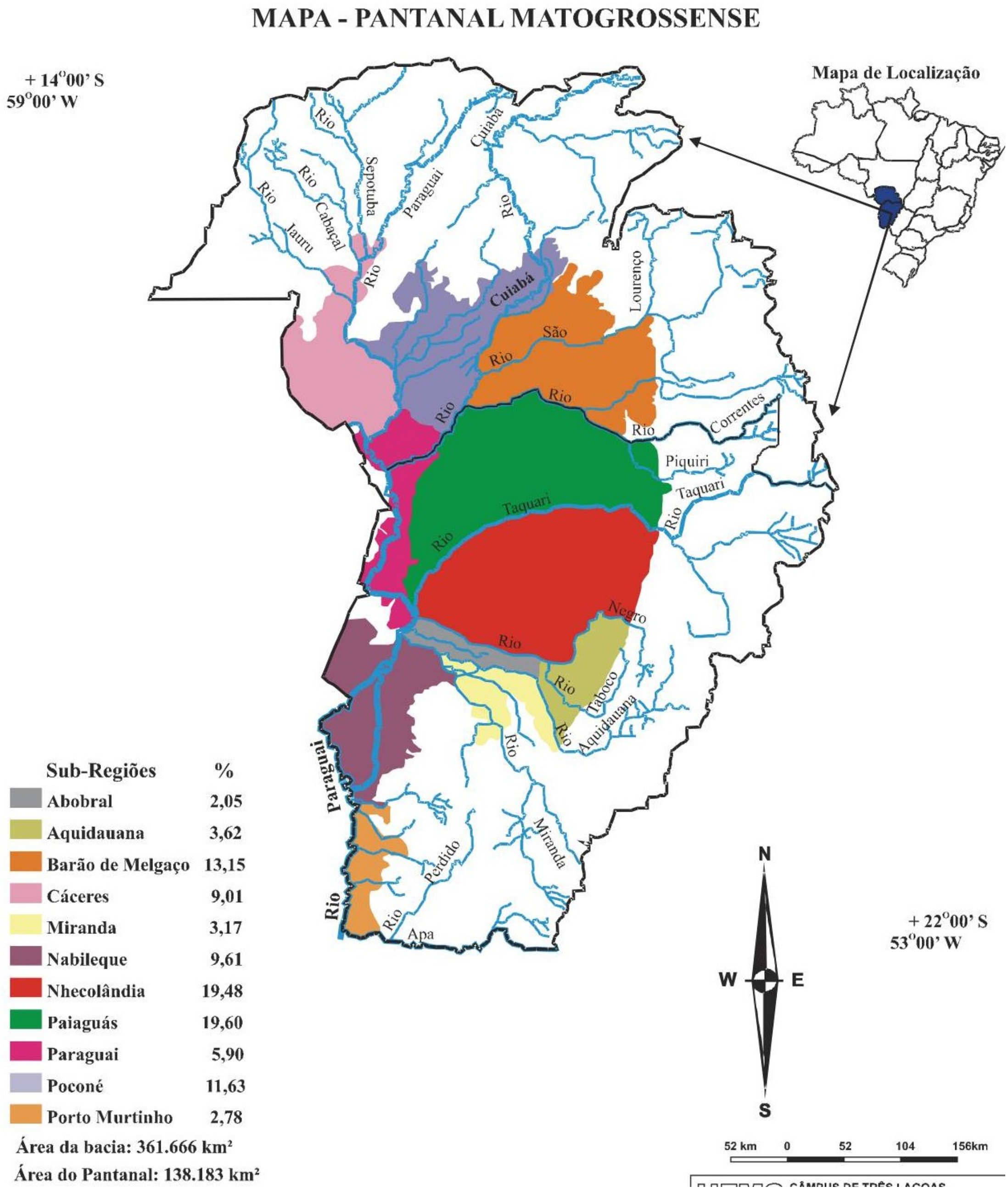


Figure 1. Pantanal of Mato Grosso and Mato Grosso do Sul-Brazil with subdivisions proposed by Silva and Abdon. Source: Silva et al (2013).

The Pantanal biome is characterized by a flooded surface, being one of the largest wetlands on the planet. Environmental organizations such as WWF and Biodiversity support program emphasize the need to preserve this ecosystem due to its natural resources. The region supports an exuberant fauna consisting of 263 species of fish, 41 of amphibians, 113 of reptiles, 463 of birds, 132 of mammals and a considerable diversity of freshwater prawns (Willink et al., 2000). An high number of species is still being described for the region as a number of systematic and biodiversity surveys are being carried out (Dos Santos et al., 2013; Junk et al., 2006; Willink et al., 2000). Pantanal offers several ecosystems services such as water retention and sedimentation, water purification, stabilization of the climate in the region, maintenance of biodiversity and quality of life for local populations (Junk et al., 2006). It has annual hydrological cycles characterized by floods and droughts, which are responsible for the region's productivity and biodiversity (Alho and Sabino, 2011). These cycles may vary according to the Pantanal region and the climatic variations of each year. Flooding favors the decomposition of submerged vegetation at the beginning of the flood and enriches the water with organic matter. Subsequently, all this water is transported to the bed of rivers and marginal lagoons (Alho et al., 2011; Alpizar et al., 2011; Calheiros, 2003; Guimarães et al., 2014; Junk et al., 2006; Pott and Pott, 2004).

2.2 Climate change

Anthropogenic changes have been causing well known effects on the climate which have been calling the attention not only of the scientific community but also of the general population (Kattwinkel et al., 2011; Lorenzoni et al., 2005; Moe et al., 2013; Noyes et al., 2009). An increase in the frequency of extreme weather events such as periods of major drought or flood and storms is expected (Moe et al., 2013). These changes have direct effects on climate components such as temperature and precipitation (Delcour and Spanoghe, 2015; Harstad, 2016; Nadal et al., 2015; Noyes et al., 2009). Changes in the precipitation patterns will also imply changes in water quality (Andrade et al., 2017; Delcour and Spanoghe, 2015). For instance oxygen depletion and increased water temperature are expected in droughts periods while intense rainfalls will increase the transport of toxic substances (such as agrochemical residues) into the aquatic environment (Delcour and Spanoghe, 2015). It is assumed that climate change can lead to shorter or longer drought periods that may decrease river dilution capacity and worsen water quality, as well as accentuate many forms of water contamination such as high levels of nutrients and chemical compounds (Marengo, 2008; Pereira et al., 2010; Ramsar, 2010). The toxicity of these chemical compounds under a scenario of environmental variability has been raising concern due to the lack of knowledge about possible interactions (stronger than additive effects between the two stressors) (Noyes et al., 2009).

Climate change combined with toxicant may thus have effects at population and community levels (Fig 2) through several processes including increased toxicant exposure due to altered environmental conditions (hydrological regimes, nutrients, pH, nitrite, etc.) and increased toxicity due to interaction with environmental components (due to climate-induced toxicant sensitivity or toxicant-induced climate susceptibility) (Moe et al., 2013).

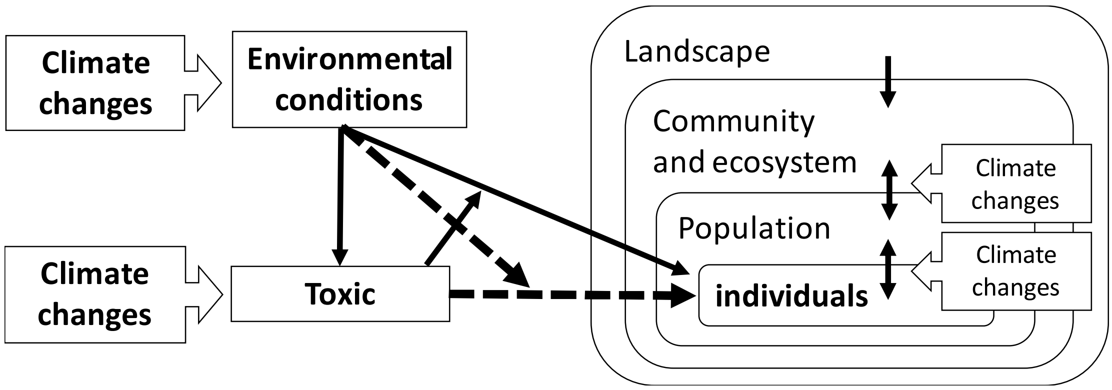


Figure 2. Combined impacts of global climate change and chemical stressors on biological organization levels. Dashed line indicates the focus of this study. Source: Adapted from Moe et al. (2013).

The fate and release of contaminants may thus be altered by climate change. Agrochemicals for instance can enter water bodies (rivers, lakes, streams, among others) by spraying, runoff, or leaching and their effects can be changed given that variations in key parameters such as pH or salinity influence the degradation rate of compounds into metabolites (Andrade, 2015; Chen et al., 2004; Dietrich and Schlatter, 1989; Maund et al., 2002; Wang et al., 2013)(Kattwinkel et al., 2011).

One of the challenges for ecotoxicology is thus to predict how the effects of changes in environmental conditions caused by climate change and environmental contaminants may interfere and affect aquatic species in unexpected ways. Studying the effects of climate change on water physic-chemical properties and how these influence existing aquatic contamination is important for correctly assess environmental risk of contaminants especially in particularly sensitive biomes with intrinsic seasonal variability as it is the case of the Pantanal region.

2.3 Climate Change in the Pantanal

Major impacts of climate change on the Pantanal are expected (Ioris et al., 2014). Projections using global climate models from the Intergovernmental Panel on Climate Change show that by the end of the century, temperatures may rise 7 °C and precipitation may decline in both summer and winter in the Pantanal (Marengo et al., 2015). The authors indicate that the water balance in the Pantanal region can be altered to drier periods and greater evaporation, however there are uncertainties, especially for rainfall (Marengo et al., 2015).

In addition to these changes, there is also a growing concern about the effects of the progress of human activities, including agriculture, on the functioning of this particular and unique ecosystem (Gomes and Barizon, 2014). The ecological risk of agricultural activities in biomes such as the Pantanal may be increased due to their particular characteristics. Each year, the annual water regime comprises a flood season and a drought period where the water is limited to the channel of the rivers, corixos, lagoons and plains (Alho, 2008; Alpizar et al., 2011; Junk et al., 2006; Pott and Pott, 2004). The slow flow of the rivers when they reach the flooding surface results in a slower stream velocity and a decrease in suspended sediment (Alpizar et al., 2011). The concentration of dissolved oxygen dramatically decreases to what follows an increase in the concentration of carbon dioxide and decrease in pH. Flood pulses with periods of high and low water make limnological parameters such as pH and nitrite vary according to the composition of the water bodies of the Pantanal region, which have a relatively small depth and are prone to these changes.

For a better understanding, Table 1 shows some values reported in the literature of pH and nitrite levels for water bodies in the Pantanal region during flood and dry periods. In a pond in the Miranda Pantanal in Mato Grosso do Sul (MS) annual variations were recorded between 6.5 and 8.2. The highest values of pH were found during the period of high water and, according to the author, may be related to the fires in the dry season that favor the entrance of alkaline substances by means of leaching of the soil with the increase of the volume of the waters (Júnior, 2013). Other studies report the opposite trend with higher pH in the dry period. For example, in the Pantanal of the Paraguay River (Corumbá-MS) the pH range was 5.57 to 7.46 (Tuiuiú Bay) and 5.54 to 7.29 (Bracinho) in the flood and in the dry, respectively (Andrade, 2011). This behavior was verified in the northern Pantanal in Mato Grosso (MT) (Silva, 2004), where variations were attributed to the reduction of water level, increased evaporation, decomposition of macrophytes and the presence of large numbers of birds in this region. Other factors resulting from human activity may influence pH variation such as cattle breeding and the construction of small dams (de Oliveira and Calheiros, 2011).

The pH plays an important role in aquatic ecosystems because it directly affects the structure of organisms (Muniz, 2010). For several species of fish, for example, the alteration of this parameter may induce reduction of reproductive, respiratory, locomotor, alimentary efficiency, as well as several cellular and molecular damages (Fromm, 1980; Kwong et al., 2014; Oliveira and Goulart, 2000).

Table 1. Chemical parameters of water (pH and nitrite) found in water bodies in the Pantanal.

Reference	Local	Dry	Full
	pH		
Arrieira et al. (2017)	Paraguay river and Miranda river	6.71	7.6
Rezende-Filho et al. (2015)	Upper Paraguay Basin		6.27 (5.23-8.24) 6.79 (6.05-7.96)
Costa et al. (2015)		6.6-69 (Bay) 9.6 (Saline) 9.9 (Salted)	
Junior 2013	Lagoon Baiazinha, Miranda-MS	6.54	8.06
Andrade 2011	Bay Tuiuiú	7.46 (6.67-8.17)	5.57 (5.34-5.76)
	Bracinho	7.29 (7.11-7.38)	5.54 (5.47-6.76)
	Nitrito (µg/L)		
Arrieira et al. (2017)	Paraguay river and Miranda river	33.3 (ammonia)	21.4 (ammonia)
Rezende-Filho et al. (2015)	Upper Paraguay Basin		46 (7.36-644) 64.4 (5.98-1520)
Junior 2013	Lagoon Baiazinha, Miranda-MS	0	170

The nitrite levels in turn are related to the nitrogen cycle. During the high waters occurs decomposition of the submerged vegetation, causing a drastic reduction of the [oxygen and ammonia release due to the microbial decomposition of organic residues and nitrogenous excretions, a natural process known as "Decoada" (Calheiros et al., 2000; Hamilton et al., 1997, 1995; Oliveira et al., 2010; Zeilhofer et al., 2016). The ammonia undergoes a process of nitrification and is directly linked to the variation of nitrite levels. In this sense, nitrite is relevant in the process of decomposition of organic matter, when the vegetation is submerged in the rivers, lagoons and bays of the Pantanal in the flood period (Silva, 2004). Higher nitrite levels in high waters were reported by Júnior (2013) (170 µg/L) and Rezende-Filho et al. (2015) (64.4 µg/L). Thus, nitrite plays an important role in the process of organic matter decomposition in rivers, lakes and bays in the Pantanal. Studies monitoring nitrite levels in water bodies of Pantanal are scarce.

Abrupt changes in parameter values such as pH or nitrite concentrations become stress factors for aquatic organisms, as each species has a tolerant range and may also interact with contaminants carried into aquatic ecosystems, increasing or decreasing their toxicity (Alpizar et al., 2011).

2.4 Agrochemicals used in the Pantanal

Economic development in Pantanal is based in agriculture, livestock, hunting, fishing, mining and tourism (Pott and Pott, 2004; Ross and Sanches, 2006). With the expansion of agricultural and livestock activity in this region, areas of forests and savannahs give rise to cultivation of soybeans, rice, corn, wheat, beans, cotton, pasture, among others (Galdino et al., 2006). These activities are characterized by the use of chemical pesticides, including several active principles to ensure good productivity, which can cause serious contamination of surface and ground water (Alpizar et al., 2011; Galdino et al., 2006; Ross and Sanches, 2006).

Chemical pesticide residues can reach the aquatic environment in two ways: transportation by rain from the areas where they are applied (eg corn or soybean crops) or by direct contact with the aquatic environment, as it is the case of rice (produced in flood areas) (Alho, 2008; Ross and Sanches, 2006). It is anticipated that this practice could lead to large ecological imbalances, given the lack of studies on the toxicity of chemical pesticides to aquatic organisms in the Pantanal region (De Carvalho Dores, 2015) and especially the influence of climate change on the toxicity of these compounds. Some monitoring studies report the presence of chemical pesticide residues in the Northeast of the

Pantanal in 68% of the surface water samples (n = 139), 87% of the rainwater samples (n = 91) and 62% of the samples of sediments (n = 26) (Laabs et al., 2002).

One of the compounds mostly used in the state of Mato Grosso do Sul is Barrage®, an emulsifiable concentrate whose active principle is cypermethrin (alpha-cyano-3-phenoxybenzyl-2,2-dimethyl-3- 2-dichlorovinyl) cyclopropane carboxylate. This formulation contains 150 grams of cypermethrin per liter and is marketed by ZOETIS - FORT DODGE - Brazil. Barrage® is used for spraying for agricultural applications, as well as in domestic applications (tick and insect control), as well as in cattle for control of flies or ticks (de Barros, 1992; Gomes et al., 2011; NPTN, 1998). Cypermethrin acts as a neurotoxin rapidly affecting the central nervous system of insects, being highly toxic to invertebrates (eg, aquatic insects and bees) and vertebrates (eg fish) (NPTN, 1998).

In a study carried out in the Pantanal of Mato Grosso do Sul, (Calheiros et al., 2006) found cypermethrin in surface water samples (mainly in Paraguay River effluents) and in sediments in other areas of the state of Mato Grosso do Sul. Cypermethrin residues were also found in five samples (n = 104) of rainwater in Lucas do Rio Verde (Mato Grosso, Brazil) at concentrations between 0.02 and 0.52 µg/L (Moreira et al., 2012). Although the concentrations detected are low, an environmental risk analysis is essential because of the high toxicity of cypermethrin to aquatic organisms and the high fragility of the Pantanal biome (Gomes and Barizon, 2014).

It is necessary to evaluate the impact of climate changes through the variation of limnological parameters, such as pH and nitrite in the cypermethrin toxicity, through the Barrage® formulation, for the aquatic organisms of the Pantanal. Previous studies showed high toxicity of Barrage® to larvae of the endemic shrimp *M. pantanalense* with a 96h-LC_{50} of 0.05 µg/L, Amazonia shrimp 96h-LC50 of 0.10 µg/L and for zebrafish 144h-LC50 of 1680 µg/L (Soares et al., 2017) (Tab. 2). Additionally, the same range of cypermethrin toxicity values were found to zebrafish (96h-LC50= 0.05 µg/L) (Sathya et al., 2014) and for shrimp *Paratya australiensis* (96h-LC50= 0.019 µg/L) (Kumar et al., 2010). Given its high toxicity, the maximum permissible concentration of cypermethrin in water is 0.09 ng/L (Crommentuijn et al., 2000).

Table 2. Effects of cypermethrin through the Barrage® formulation on crustacean and fish species. Source: Soares et al., 2017

Species	Time (hours)	Endpoint	L(E)C10 (μ/L)	L(E)C50 (μ/L)	Changes
Macrobrachium pantanalense	**48**	Survival	0.059 (0.02)	0.19 (0.03)	Reduction of carapace length and development (zoea phases)
	96	Survival	0.024 (0.01)	0.05 (0.01)	
Macrobrachium amazonicum	**48**	Survival	0.17 (0.06)	0.63 (0.09)	Reduction of carapace length, development (zoea phases) and number of lipid droplets
	96	Survival	0.04 (0.01)	0.10 (0.01)	
Danio rerio	**72**	Hatching	571 (226)	3210 (588)	Tail malformations, pericardial edema, equilibrium (unbalance, sideways) tremors, heartbeat, larvae length. Hatching was stimulated for intermediate concentrations and high concentrations delayed hatching
	144	Mortality	561 (142)	1680 (689)	
	144	Edema	236 (156)	254 (50.0)	
	144	Tremors	94.0 (36.0)	1490 (1106)	
	144	Side-ways[a]	135 (61.0)	218 (28.0)	

[a] Larvae positioned side-ways (extreme loss of equilibrium).

2.5 Ecotoxicology

Ecotoxicology is the science that studies the effects of chemicals in the environment, understanding and predicting the effects of pollutants on living beings and natural communities, as well as the interaction of chemicals, living beings and the environment (Knie and Lopes, 2004; Zagatto and Bertoletti, 2008). Ecotoxicological tests fall in two categories: acute and chronic. The acute tests are short-term tests and detect the sudden and usually irreparable effects to organisms; the chronic involve longer exposures, evaluating damages that appear at lower concentrations (Knie and Lopes, 2004). In the last years endpoints more sensitive which respond better to effects of low concentrations of pollutants have been sought in order to allow better prevision of risks in ecological relevant scenarios. Individual and sub-individual parameters under the general denomination of biomarkers have been increasingly used (Yancheva et al., 2016) and include histological, physiological, biochemical, genetic and behavioral responses (Cristina Fossi and Marsili, 1997; Fossi, 1998).

Histological changes can be evaluated as biomarkers since they are very sensitive to the effects of the chemical compounds, which allows to evaluate changes in the selected organs and to measure more precisely the contamination of the aquatic environment (Yancheva et al., 2016). The evaluation of changes in gills, for example, is an important procedure to estimate the impact of chemicals in the aquatic environment, because it is directly in contact with water and because of its high permeability (Maharajan et al., 2015). As well as for behavioral biomarkers it aims to understand how exposure to contaminants can alter the individual fitness and persistence of populations of non-target aquatic organisms (Dell'Omo, 2002). Behavioral endpoints are early warning tools that have been increasingly successful in protecting and / or restoring aquatic ecosystems (Hellou, 2011). Interactions between the body's internal mechanisms and external environmental or social pressure will link physiological and biochemical changes due to stress, putting health and survival at risk (Beauvais et al., 2000; Hellou, 2011). In addition, the behavioral effects can be cumulative and can be classified according to the response time in: 1- avoid / flee; 2- balance, ability to straighten, response of fear and feeding; or locomotion, mating, memory learning, protection of the pups, breathing, among others (Hellou, 2011), and can be altered according to the species. In this way, behavioral ecotoxicology can help develop sensitive tools to investigate toxic effects and the broader the studies with different species and chemicals the greater the prediction / protection of risk.

"Model organisms" to be used in ecotoxicity testing must have certain characteristics such as: the possibility of being easily cultivated or kept in the laboratory and balanced sensitivity that can safely react only to the real toxic effects, since hypersensitive organisms can cause false results caused by marginal phenomena, such as changes in temperature. Organisms that have strong physiological defense mechanisms are also inadequate for this type of study because they may present underestimated results (Knie and Lopes, 2004; Zagatto and Bertoletti, 2008). Given all these factors, choosing the model organism in a risk assessment is a key step. Many studies point to a greater sensitivity of endemic organisms, since the use of more generalized model organisms may, in certain cases, result in an erroneous estimate of environmental risk (Raj Pandrangi, Michael Petras, Steve Ralph, 1995; Zagatto and Bertoletti, 2008).

Studies using *Macrobrachium* species (*M. amazonicum*, *M. rosenbergii* and *M. olfersii*) in toxicological tests have been carried out by several authors (Barbieri et al., 2013; Camacho-sánchez MI, 2007; Chang et al., 2013; Maria de Medeiros et al., 2001; Revathi et al., 2014; Sánchez and Delgado, 2003; Vinícius et al., 2014), however, these species are not endemic to the Pantanal region and have a distinct life cycle and biology, with larval cycle phases occurring in estuarine regions. These species, after being transformed into post-larvae, begin to migrate to the interior of the rivers and continue their development in fresh water (Magalhães, 2003). Therefore, the use of a native species for toxicity evaluation allows greater accuracy and confidence in the results of ecotoxicological evaluations.

Taking into account that the studies on the contaminant impacts on species belonging to the Pantanal Basin are scarce, in this work, two shrimp species of the genus *Macrobrachium* were selected: *M. pantanalense* an endemic shrimp of Pantanal and the Amazonian shrimp *M. amazonicum.* Moreover the zebrafish (*Danio rerio*) was also selected as a model species widely used in ecotoxicology. As behavioral endpoints studied in zebrafish revealed to be very useful tools for assessment of Barrage® effects, methodologies were adapted for shrimp, using as model species the *Palaemon varians*.

2.6 Shrimps of the genus Macrobrachium

The current taxonomic description is as follows (WoRMS, 2018a):

Kingdom: Animalia
Phylum: Arthropoda
Class: Crustacea
Subclass: Malacostraca
Superordinate: Eucarida
Order: Decapoda (Latreille, 1802)
Suborder: Caridea (Dana, 1852)
Superfamily: Palaemonidae (Rafinesque, 1815)
Family: Palaemonidae (Rafinesque, 1815)
Subfamily: Palaemonidae (Rafinesque, 1815)
Genre: *Macrobrachium* (Bate, 1868)
Species: *Macrobrachium amazonicum* (Heller, 1862)
Macrobrachium pantanalense (Dos Santos, Hayd & Anger, 2013)

The species of shrimp that occurs in Pantanal, *M. pantanalense,* was for a long time identified as *M. amazonicum*, but recently the two shrimps were distinguished and *M. pantanalense* classified as a new species, with the main differences being found in the morphology and color patterns of both sexes (Fig. 3). *Macrobrachium pantanalense* is transparent, has brown spots around the body and reduced size (Dos Santos et al., 2013).

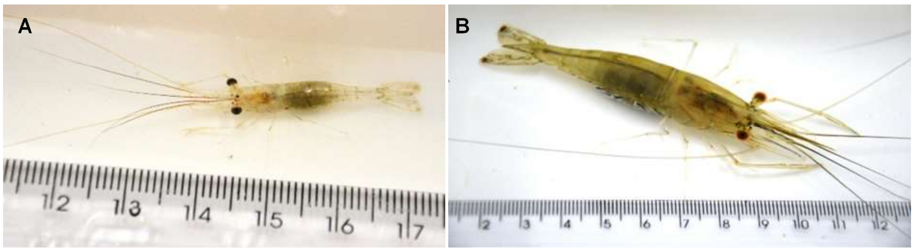


Figure 3 - *Macrobrachium pantanalense* (A) and *Macrobrachium amazonicum* (B) ovigerous female (Source: personal).

This species exhibits sexual dimorphism, males are smaller than females (Hayd and Anger, 2013). These authors indicate that males initiate sexual activity with body size close to 19 mm, and for females the onset of maturity is observed at a minimum body size of ovigerous females of 29.8 mm. The average size of adults of the *M. pantanalense* species varies between 30 - 50 mm for males and 50 - 70 mm for females.

The life cycle is divided into egg, larval stage, juvenile and adult. The females' fecundity depends on their size and can reach a limit of 676 eggs (Hayd and Anger, 2013; Vercesi and Hayd, 2015). The embryonic development of the larvae lasts about 19 to 23 days, and goes through 11 larval stages (zoea phases), presenting in this period positive phototaxis and planktonic feeding habit (Fig. 4). Then, larvae undergo metamorphosis reaching the post-larva stage, where they begin to present benthic habits and are able to swim freely in the water column (Hayd and Anger, 2013).

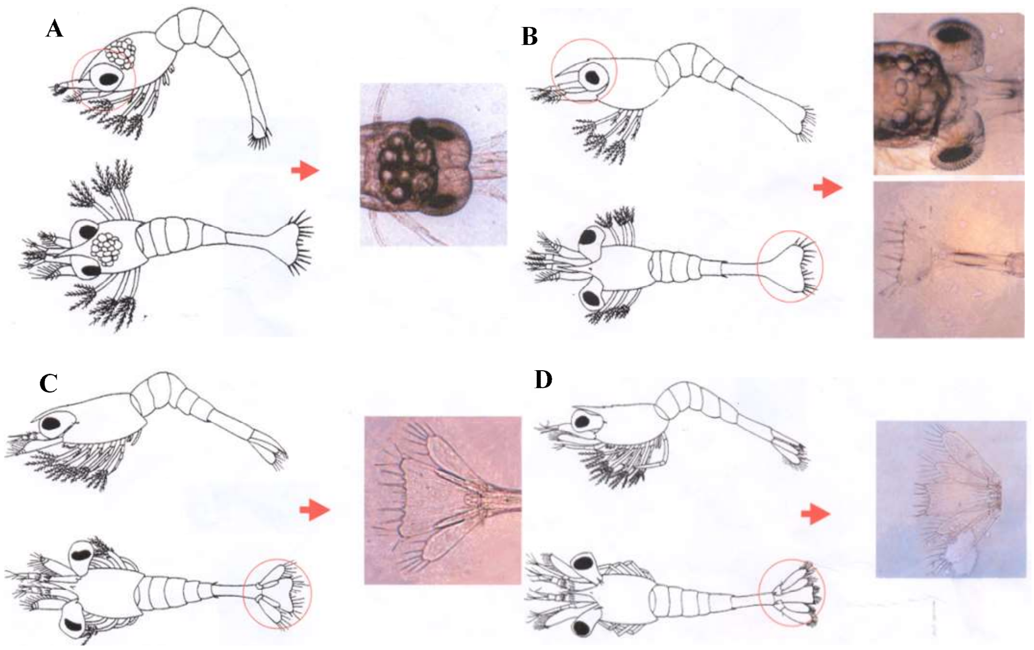


Figure 4 - Macrobrachium zoea stages: zoea I, sessile eyes (A); zoea II, pedunculated eyes; absent uropods (absence of endopodite and exopodite), only the télson appears in the last segment (B); zoea III, uropods constituted by developed exopodites, with bristles and rudimentary, nude endopodites, on the telson (C); zoea IV, exopodites and endopodites of developed and bristle (D) uropods (Vega-Perez, 1984).

This species has larval and adult development in freshwater, inhabiting inland waters (Dos Santos et al., 2013), however, studies show that salinity may reduce the time of larval development of this species when grown in the laboratory. Vercesi (2014) found that larvae of *M. pantanalense* cultivated in starvation present higher survival in salinity 5.

This species is distributed in the Paraguay River Basin, having a key role in the trophic chains of this ecosystem. Studies by the research group show that this endemic shrimp, presents high sensitivity to toxic compounds when compared to other model species (Soares et al., 2017) supporting its use as a model species in studies related to the Pantanal region.

M. amazonicum, is a species of shrimp that is widely distributed in South America (Fig. 6), with a higher occurrence in the northern and northeastern basins of Brazil (Holthuis 1952; Coelho e Ramos-Porto 1984). Unlike Pantanal shrimp, the Amazonian shrimp has great morphological variability among adult males. Differences are mainly in the size and color of the second pair of pereopods (Fig 5) and translates a social structure of dominance of males (Moraes-Riodades and Valenti, 2004).

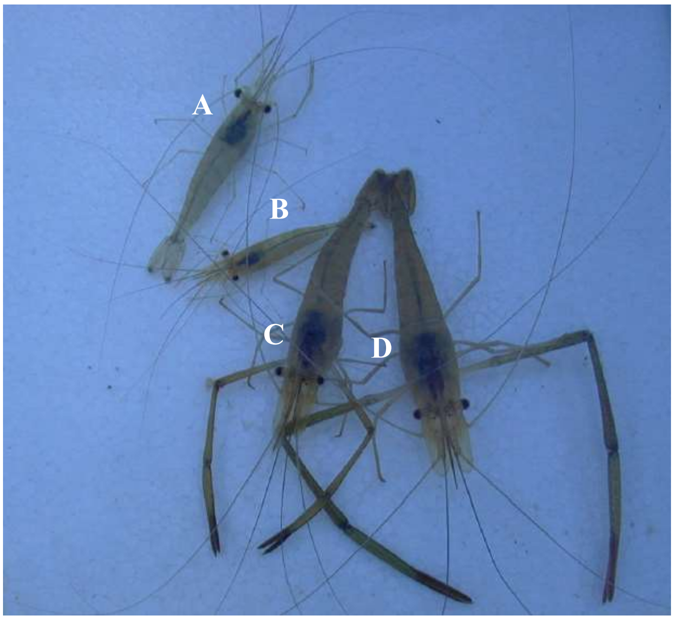


Figure 5 - *Macrobrachium amazonicum*, A-TC, B-CC, C-GC1 and D-GC2 morphotypes of shrimp males. Source: Hayd (2007)

The larval development of *M. amazonicum* is totally dependent on salinity, being tolerant to the great variation of salinity of estuary regions (Araujo, 2005; Maciel and Valenti, 2009; Moraes-Valenti and Valenti, 2010). The larvae go through 9 larval stages (zoea phases). In laboratory, in optimal culture conditions (salinity 10 to 12 and temperature 28ºC) larval cycle and metamorphosis into decapodite (juvenile phase) can occur in 18 to 19 days (Anger et al., 2009; Vetorelli, 2008).

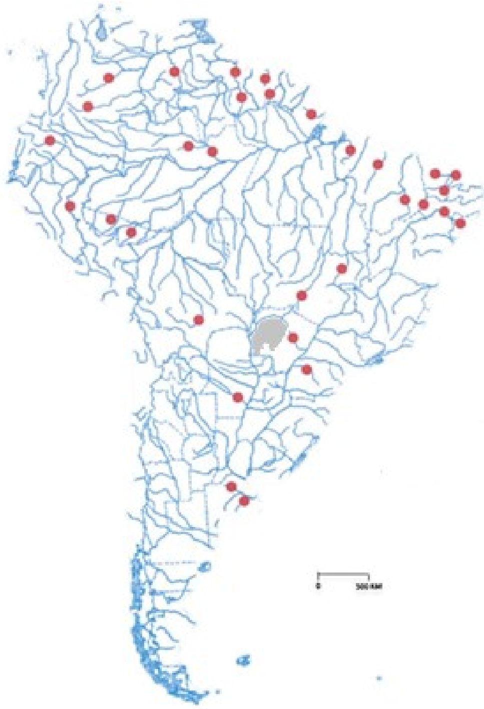


Figure 6- Geographical distribution of *Macrobrachium pantanalense* ( ) and *Macrobrachium amazonicum* ( ) (adapted from Melo, 2003).

2.7 *Palaemon varians*

The taxonomic description is as follows (WoRMS 2018):

Kingdom: Animalia

Phylum: Arthropoda

Class: Crustacea

Subclass: Malacostraca

Superordinate: Eucarida

Order: Decapoda (Latreille, 1802)

Suborder: Caridea (Dana, 1852)

Superfamily: Palaemonidae (Rafinesque, 1815)

Family: Palaemonidae (Rafinesque, 1815)

Subfamily: Palaemonidae (Rafinesque, 1815)

Genre: *Palaemon* Weber, 1795

Species: *Palaemon varians* Leach, 1813

The *P. varians* shrimp species occurs in estuarine regions distributed from the northwestern European, Mediterranean and Atlantic coast of Morocco (Dolmen et al. 2004). The general distribution comprises Europe (Norway, Sweden, Denmark, Germany, Ireland, United Kingdom, Netherlands, Belgium, France, Portugal, Spain), NW Africa (Morocco, Algeria, Tunisia) (Christodoulou et al., 2016). (Fig. 7).

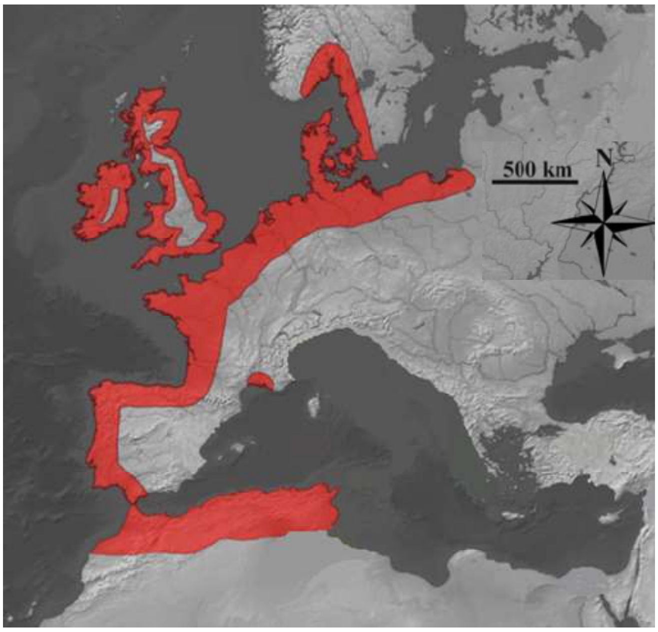


Figure 7 - Geographical distribution of *Palaemon varians* ( ) in the broader Mediterranean region Source: adapted from Christodoulou et al. (2016).

The shrimps of this species are adapted to a wide range of temperature and salinity conditions (Cottin et al., 2010; Palma et al., 2009). They can develop and reproduce in low salinity environments (close to 0), as well as high salinities (35), although they do not occur in freshwater environments (González-Ortegón e Cuesta 2006; Oliphant e Thatje 2014). Shrimps of this species are relatively small in size, translucent in color, and sexual dimorphism occurs by identifying an appendix on the second pair of pleopods in adult males (Calado, 2008; Rodríguez et al., 1993; Ruppert and Barnes, 1993). The sexual maturity of males is reached with 16 mm and for females 22 mm respectively (Antonopoulou e Emson 1992; Gelin e Souty-Grosset 2006). Females have fecundity between 85 and 412 embryos that are incubated in the abdominal cavity protected by pleopods with a duration of approximately 15 days (Antonopoulou e Emson 1992; Rodríguez et al. 1993) (Fig. 8).

Figure 8- *Palaemon varians*, ovigerous adult female; embryos at the end of the incubation period. Source: (Oliphant et al., 2013)

Development is relatively fast (Palma et al. 2008), the larvae pass through 5 larval stages (zoea fazes) until they reach decapodite phase with juvenile-like morphological structures (Fincham, 1979) (Fig. 9). Due to this accelerated development (that lasts on average 12 days) the species is classified as species of abbreviated development (Fincham 1979; Oliphant et al. 2011; Oliphant et al. 2013; Oliphant e Thatje 2014). After hatching larvae have positive phototaxis, being attracted by light, swimming head-first with the help of the thoracic appendages (Anger, 2001). The larval development of *P. varians* can be carried out at a wide range of salinity between 5 and 42 (Antonopoulou e Emson, 1988; Dolmen et al., 2004; Gelin e Souty-Grosset, 2006) and temperature between 10 and 30º C (Palma et al., 2009; Oliphant et al., 2013).

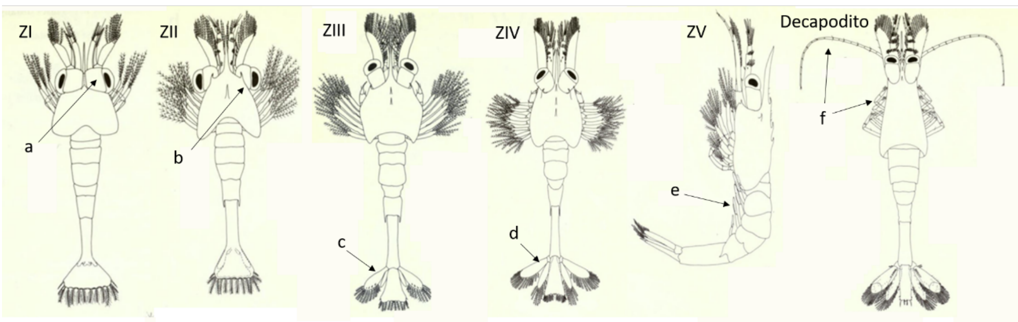


Figure 9- Stages of larval development (zoea phases) of *Palaemon varians*: ZI - Decapodid. (a) sessile eyes; (b) pedunculate eyes; (c) endopodite of the uropode developed; (d) endopodite and exopodite of the uropode developed; (e) developed pleopods; (f) antenna and pereiopods developed. Source: Fincham (1979).

P. varians prawns have been widely used as model organisms for environmental risk assessments due to their easy cultivation and maintenance in the laboratory, as well as their fast larval development and easy identification (Jorge Palma et al., 2008; Cottin et al., 2012; Rainbow e Smith, 2013; New et al., 2014; Correia et al., 2016; Pavlaki et al., 2016).

2.8 Zebrafish

The current taxonomic description is as follows:

Kingdom: Animalia
Filo: Chordata
Class: Actinopterygii
Order: Cypriniformes
Family: Cyprinidae
Subfamily: Rasborinidae
Genre: *Danio*
Species: *Danio rerio* (Hamilton-Buchanan, 1822)

Danio rerio, known as zebrafish, has been widely used in scientific research due to their easy cultivation, reproduction and high egg production in laboratory (Beliaeva et al., 2010; Zhang et al., 2015). The genome of this species was completely sequenced and for 70% of the genes a human ortholog as identified (Howe et al., 2013) turning zebrafish in a model of human diseases (Williams et al., 2016).

The embryonic fish toxicity test (FET) is an alternative for acute fish testing for animal welfare, since testing for this group of organisms is a mandatory component in ecotoxicity assessments (Lammer et al., 2009). The transparency of zebrafish eggs is what makes it a model organism to evaluate sub-lethal endpoints, including edema, tail deformation, developmental delay, such as hatching, among other parameters and advantages by the easy monitoring of embryonic development (Beliaeva et al., 2010; Lammer et al., 2009; Zhang et al., 2015). Other advantages of using FET are cost-related, relatively cost-effective for easy maintenance over larger vertebrate tests, reduced chemical dilution, and reduced time trials (Lammer et al., 2009). All these characteristics of zebrafish embryos make us excellent models of animal experimentation for the understanding of mechanisms of toxicity and possible long-term adverse effects (Lammer et al., 2009).

The geographical distribution of *D. rerio* is listed in the literature for all South and Southeast Asia, including India, Bangladesh and Nepal, as well as for the entire Indian continent (Fig. 10) (Engeszer et al., 2007; Spence et al., 2007, 2006). The fish of this species have an average length between 4 and 5 cm and can be grown in larger numbers in aquaria in the laboratory guaranteeing reproduction all year round (ABNT, 2011; Schneider et al 2009).

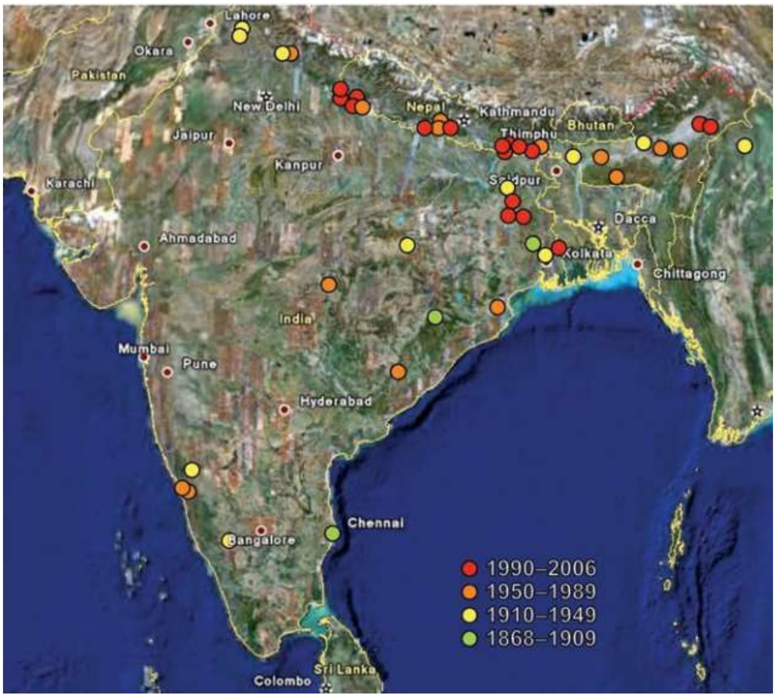


Figure 10 - Geographical distribution of *Danio rerio*. Source: Engeszer et al. (2007)

The differentiation of male and female is easily identifiable, where males have yellowish belly, are thinner and longer and have large anal fin, females are silvery, fatter, small anal fin and small genital papilla (Oliveira, 2009) (Fig. 11).

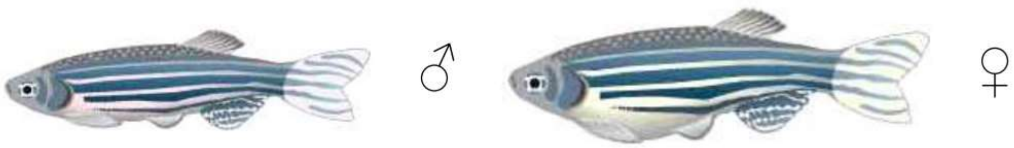


Figure 11- Representation of male and female Zebrafish. Source: Oliveira (2009)

Sexual maturity is reached at 3 months of life. Egg production is about 200-300 eggs per spawning and fertilization is external, the eggs are transparent allowing the easy monitoring of the embryonic development (Fig. 12) (Kimmel et al., 1995; Spence et al., 2007). Usually the larvae hatch after 48 to 72 h and on the fifth day after fertilization, most of the tissues are developed (Kimmel et al., 1995). Larval phase lasts up to 30 days, juveniles up to 90 days and adults usually live up to 2 years.

The short life cycle, reduced size, rapid embryonic development, transparent and non-adherent eggs, good captive breeding, and sequenced genome make it an excellent model organism for ecotoxicological trials.

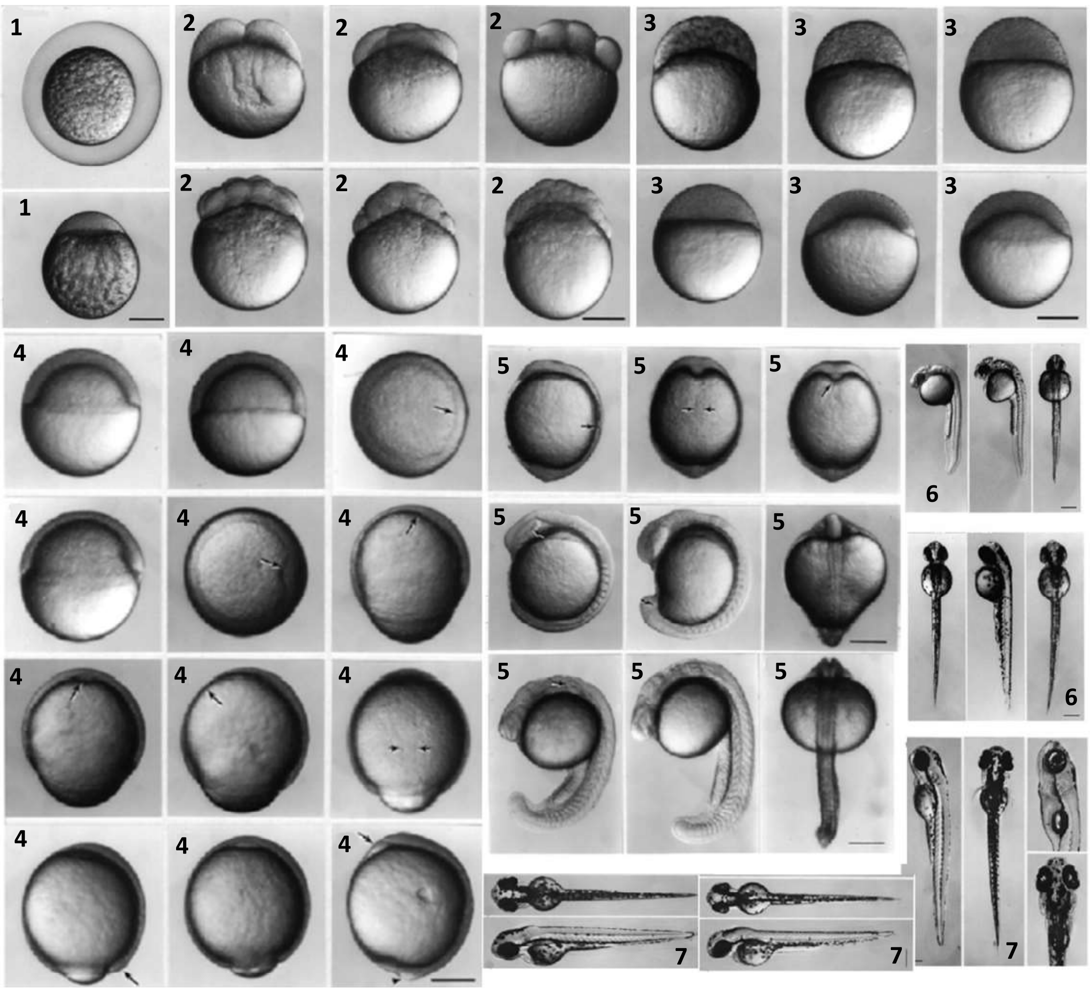


Figure 12- Zebrafish embryo development stages (*Danio rerio*): 1-zygote, 2-cleavage, 3-blastula, 4-gastrula, 5-segmentation, 6-pharyngula, 7-hatching of the early larvae. Source: Adapted from Kimmel et al., (1995).

3 References

Alho, C., Fischer, E., Oliveira-Pissini, L., Santos, C., 2011. Bat-species richness in the Pantanal floodplain and its surrounding uplands. Brazilian J. Biol. 71, 311–320. https://doi.org/10.1590/S1519-69842011000200010

Alho, C., Sabino, J., 2011. A conservation agenda for the Pantanal's biodiversity. Brazilian J. Biol. 71, 327–335. https://doi.org/10.1590/S1519-69842011000200012

Alho, C.J.R., 2008. Biodiversity of the Pantanal: response to seasonal flooding regime and to environmental degradation. Brazilian J. Biol. 68, 957–966. https://doi.org/10.1590/S1519-69842008000500005

Alpizar, F., Carlsson, F., Naranjo, M.A., 2011. The effect of ambiguous risk, and coordination on farmers' adaptation to climate change — A framed field experiment. Ecol. Econ. 70, 2317–2326. https://doi.org/10.1016/j.ecolecon.2011.07.004

Andrade, M., 2011. O fenômeno da decoada no Pantanal do rio Paraguai, Corumbá/MS: alterações dos parâmetros limnológicos e efeitos sobre os macroinvertebrados bentônicos. Doctoral dissertation, Universidade de São Paulo.

Andrade, T. de S., 2015. Effects of environmental factors on the toxicity of pesticides to zebrafish embryos. Doctoral Thesis, Universidade de Aveiro.

Andrade, T.S., Henriques, J.F., Almeida, A.R., Soares, A.M.V.M., Scholz, S., Domingues, I., 2017. Zebrafish embryo tolerance to environmental stress factors—Concentration–dose response analysis of oxygen limitation, pH, and UV-light irradiation. Environ. Toxicol. Chem. 36, 682–690. https://doi.org/10.1002/etc.3579

Anger, K., 2001. The Biology of Decapod Crustacean Larvae, 14th ed. Lisse: AA Balkema Publishers.

Antonopoulou, E., Emson, R., 1988. The combined effects of temperature and salinity on survival, moulting and motamorphosis of the larval stages of the three species of palaemonid prawns, in: Reproduc- Tion, Genetics and Distributions of Marine Organisms: Proc 23rd Eur Mar Biol Symp, Swansea. p. 339−348.

Antonopoulou, E., Emson, R.H., 1992. Aspects of the population dynamucs of *Palaemonetes varians* (Leach), in: Marine Eutrophication and Population Dynamics: 25th European Marine Biology Symposium. Ferrara, pp. 157–164.

Araujo, M.C. De, 2005. Efeitos da salinidade, luminosidade e alimentação na larvicultura do camarão-da-amazônia, *Macrobrachium amazonicum*. Tese de doutorado. Disponivel em: http://hdl.handle.net/11449/144136.

Arrieira, R.L., Schwind, L.T.F., Bonecker, C.C., Lansac-Tôha, F.A., 2017. Temporal dynamics and environmental predictors on the structure of planktonic testate amoebae community in four Neotropical floodplains. J. Freshw. Ecol. 32, 35–47. https://doi.org/10.1080/02705060.2016.1236758

Barbieri, E., Moreira, P., Luchini, L.A., Hidalgo, K.R., Muñoz, A., 2013. Assessment of acute toxicity of carbofuran in *Macrobrachium olfersii* (Wiegmann, 1836) at different temperature levels. Toxicol. Ind. Health. https://doi.org/10.1177/0748233713484655

Beauvais, S.L., Jones, S.B., Brewer, S.K., Little, E.E., 2000. Physiological measures of neurotoxicity of diazinon and malathion to larval rainbow trout (*Oncorhynchus mykiss*) and their correlation with behavioral measures. Environ. Toxicol. Chem. 19, 1875–1880. https://doi.org/10.1002/etc.5620190722

Beliaeva, N.F., Kashirtseva, V.N., Medvedeva, N. V, Khudoklinova, I.I., Ipatova, O.M., Archakov, A.I., 2010. Zebrafish as a model organism for biomedical studies. Biomed. Khim. 56, 120–31.

Calado, R., 2008. Marine Ornamental Shrimp: Biology, Aquaculture and Conservation, 1st ed. Oxford, Wiley-Blackwell.

Calheiros, D.F., 2003. Influência do pulso de inundação na composição isotópica (13 C e 15 N) das fontes primárias de energia na planície de inundação do rio Paraguai (Pantanal - MS). Universidade de São Paulo.

Calheiros, D.F., Dores, E.F.G., Oliveira, M.D. de, 2006. Limnologia: estudo da qualidade física e química das águas dos rios, córregos e lagos e de suas relações ecológicas com os organismos aquáticos. Ecotoxicologia: estudo dos efeitos das substâncias tóxicas nos organismos (biota) e na qualidade ambiental. ADM - Artig. Divulg. na Mídia, Embrapa Pantanal 1–4.

Calheiros, D.F., Seidl, A.F., Ferreira, C.J., 2000. Participatory research methods in environmental science: local and scientific knowledge of a limnological phenomenon in the Pantanal wetland of Brazil. J. Appl. Ecol. 37, 684–696.

Camacho-sánchez MI, 2007. Bioconcentración y toxicidad de metales en el langostino

Macrobrachium rosenbergii (de Man). Rev. Toxicol. 24, 14–17.

Chang, C.C., Rahmawaty, A., Chang, Z.W., 2013. Molecular and immunological responses of the giant freshwater prawn, *Macrobrachium rosenbergii*, to the organophosphorus insecticide, trichlorfon. Aquat. Toxicol. 130–131, 18–26. https://doi.org/10.1016/j.aquatox.2012.12.024

Chen, C.Y., Hathaway, K.M., Folt, C.L., 2004. Multiple stress effects of Vision® herbicide, ph, and food on zooplankton and larval amphibian species from forest wetlands. Environ. Toxicol. Chem. 23, 823. https://doi.org/10.1897/03-108

Christodoulou, M., Anastasiadou, C., Jugovic, J., Tzomos, T., 2016. Freshwater Shrimps (Atyidae, Palaemonidae, Typhlocarididae) in the Broader Mediterranean Region: Distribution, Life Strategies, Threats, Conservation Challenges and Taxonomic Issues, in: A Global Overview of the Conservation of Freshwater Decapod Crustaceans. Springer International Publishing, Cham, pp. 199–236. https://doi.org/10.1007/978-3-319-42527-6_7

Coelho, P.A., Ramos-Porto, M., 1984. Camarões de água doce do Brasil: distribuição geográfica. Rev. Bras. Zool. 2, 405–410. https://doi.org/10.1590/S0101-81751984000200014

Correia, M., Palma, J., Andrade, J.P., 2016. Growth performance of the early life stages of broad-nosed pipefish, *Syngnathus typhle* (L.) fed different live or frozen diets. Aquac. Res. 47, 1652–1660. https://doi.org/10.1111/are.12635

Costa, M., Telmer, K.H., Evans, T.L., Almeida, T.I., Diakun, M.T., 2015. The lakes of the Pantanal: inventory, distribution, geochemistry, and surrounding landscape. Wetl. Ecol. Manag. 23, 19–39. https://doi.org/10.1007/s11273-014-9401-3

Cottin, D., Brown, A., Oliphant, A., Mestre, N.C., Ravaux, J., Shillito, B., Thatje, S., 2012. Sustained hydrostatic pressure tolerance of the shallow water shrimp *Palaemonetes varians* at different temperatures: Insights into the colonisation of the deep sea. Comp. Biochem. Physiol. 162, 357–363. https://doi.org/10.1016/j.cbpa.2012.04.005

Cottin, D., Shillito, B., Chertemps, T., Thatje, S., Léger, N., Ravaux, J., 2010. Comparison of heat-shock responses between the hydrothermal vent shrimp *Rimicaris exoculata* and the related coastal shrimp *Palaemonetes varians*. J. Exp. Mar. Bio. Ecol. 393, 9–16. https://doi.org/10.1016/j.jembe.2010.06.008

Cristina Fossi, M., Marsili, L., 1997. The use of non destructive biomarkers in the study of marine mammals. Biomarkers 2, 205–216. https://doi.org/10.1080/135475097231571

Crommentuijn, T., Sijm, D., de Bruijn, J., van Leeuwen, K., van de Plassche, E., 2000. Maximum permissible and negligible concentrations for some organic substances and pesticides. J. Environ. Manage. 58, 297–312. https://doi.org/10.1006/jema.2000.0334

de Barros, A.T.M., 1992. Recomendações para controle da mosca-dos-chifres no Pantanal. Embrapa, Centro de Pesquisa Agropecuária do Pantanal (Corumba, MS).

Technical communication, p. 4. ISSN: 0102e8316 URL: https://www.infoteca.

cnptia.embrapa.br/infoteca/bitstream/doc/787742/1/COT10.pdf. Accessed April 2018.

De Carvalho Dores, E.F.G., 2015. Pesticides in the Pantanal. Springer Int. Publ. Switz. 1–12. https://doi.org/10.1007/698_2015_356

de Oliveira, M.D., Calheiros, D.F., 2011. Qualidade da Água em Agroecossistemas do Pantanal: Sub-regiões da Nhecolândia e Poconé. Embrapa Pantanal-Boletim Pesqui. e Desenvolv. BP109.

Delcour, I., Spanoghe, P., 2015. Literature review: Impact of climate change on pesticide use. Food Res. Int. 68, 7–15. https://doi.org/10.1016/j.foodres.2014.09.030

Dell'Omo, G., 2002. Behavioural ecotoxicology. John Wiley & Sons.

Dietrich, D., Schlatter, C., 1989. Aluminium toxicity to rainbow trout at low pH. Aquat. Toxicol. 15, 197–212. https://doi.org/10.1016/0166-445X(89)90036-2

Dolmen, D., Hindley, J., Kleiven, E., 2004. Distribution of *Palaemonetes varians* (Leach) (Crustacea, Decapoda) in relation to biotope and other caridean shrimps in brackish waters of southern Norway and southwestern Sweden. Sarsia 89, 8–21. https://doi.org/10.1080/00364820310003244

Dos Santos, A., Hayd, L., Anger, K., 2013. A new species of *Macrobrachium* Spence Bate, 1868 (Decapoda, Palaemonidae), *M. pantanalense*, from the Pantanal, Brazil. Zootaxa 3700, 534–546. https://doi.org/http://dx.doi.org/10.11646/zootaxa.3700.4.2

Engeszer, R.E., Patterson, L.B., Rao, A.A., Parichy, D.M., 2007. Zebrafish. Spring 4, 21–40. https://doi.org/http://doi.org/10.1089/zeb.2006.9997

Fincham, A.A., 1979. Larval development of British prawns and shrimps (Crustacea, Decapoda, Natantia). 2. *Palaemonetes* (*Palaemonetes*) *varians* (Leach, 1814) and morphological variation. Bull. Br. Museum Nat. Hist. 35, 163–182.

https://doi.org/10.3366/anh.1992.19.3.426

Fossi, M.C., 1998. Biomarkers as Diagnostic and Prognostic Tools for Wildlife Risk Assessment: Integrating Endocrine-Disrupting Chemicals. Toxicol. Ind. Health 14, 291–309. https://doi.org/10.1177/074823379801400118

Fromm, P.O., 1980. A review of some physiological and toxicological responses of freshwater fish to acid stress. Environ. Biol. Fishes 5, 79–93. https://doi.org/10.1007/BF00000954

Galdino, S., Vieira, L.M., Pellegrin, L.A., 2006. Impactos Ambientais e Socioeconômicos na Bacia do Rio Taquari - Pantanal, 1º. ed. Embrapa Pantana, Corumbá.

Gelin, A., Souty-Grosset, C., 2006. Species identification and ecological study of the genus *Palaemonetes* (Decapoda: Caridea) in the French Mediterranean. J. Crust. Biol. 26, 124–133. https://doi.org/10.1651/C-2487.1

Gomes, A., Koller, W., Barros, A., 2011. Susceptibility of *Rhipicephalus* (*Boophilus*) *microplus* to acaricides in Mato Grosso do Sul, Brazil. Ciência Rural 41, 1447–1452. https://doi.org/http://dx.doi.org/10.1590/S0103-84782011005000105

Gomes, M.A.F., Barizon, R.R.M., 2014. Panorama da Contaminação Ambiental por Agrotóxicos e Nitrato de Origem Agrícola no Brasil: Cenário 1992/2011 35.

González-Ortegón, E., Cuesta, J.A., 2006. An illustrated key to species of *Palaemon* and *Palaemonetes* (Crustacea: Decapoda: Caridea) from European waters, including the alien species *Palaemon macrodactylus*. J. Mar. Biol. Assoc. UK 86, 93. https://doi.org/10.1017/S0025315406012896

Guimarães, E., Manoel, P.S., Trevelin, C.C., 2014. Pantanal: fauna, flora e paisagens, 1º. ed, Coleção PROEX Digital (UNESP). Disponivel em: http://hdl.handle.net/11449/126247. Cultura Acadêmica, São Paulo: Cultura Acadêmica.

Hamilton, S.K., Sippel, S.J., Calheiros, bora F., Melack, J.M., 1997. An anoxic event and other biogeochemical effects of the Pantanal wetland on the Paraguay River. Limnol. Ocean. 42, 257–272.

Hamilton, S.K., Sippel, S.J., Melack, J.M., 1995. Oxygen depletion and carbon dioxide and methane production in waters of the Pantanal wetland of Brazil. Biogeochemistry 30, 115–141. https://doi.org/10.1007/BF00002727

Harstad, B., 2016. The dynamics of Climate Agreements. J. Eur. Econ. Assoc. 14, 719–752. https://doi.org/10.1111/jeea.12138

Hayd, L., Anger, K., 2013. Reproductive and morphometric traits of *Macrobrachium amazonicum* (Decapoda: *Palaemonidae*) from the Pantanal, Brazil, suggests initial speciation. Rev. Biol. Trop. 61, 39–57.

Hellou, J., 2011. Behavioural ecotoxicology, an “early warning” signal to assess environmental quality. Environ. Sci. Pollut. Res. 18, 1–11. https://doi.org/10.1007/s11356-010-0367-2

Holthuis, L.B., 1952. A general revision of the *Palaemonidae* (crustacea Decapod Natantia) of the Americas. II. The subfamily *Palaemonidae*. Disponivel em: http://hdl.handle.net/1969.3/20038.

Howe, K. et al., 2013. The zebrafish reference genome sequence and its relationship to the human genome. Nature 496, 498–503. https://doi.org/10.1038/nature12111

Ioris, A.A.R., Irigaray, C.T., Girard, P., 2014. Institutional responses to climate change: opportunities and barriers for adaptation in the Pantanal and the Upper Paraguay River Basin. Clim. Change 127, 139–151. https://doi.org/10.1007/s10584-014-1134-z

Júnior, R.C.M., 2013. Avaliação dos parâmetros físicos e químicos da lagoa Baiazinha, Pantanal de Miranda-MS. Dissertação de mestrado. Universidade Estadual do Mato Grosso do Sul.

Junk, W.J., Da Cunha, C.N., Wantzen, K.M., Petermann, P., Strüssmann, C., Marques, M.I., Adis, J., 2006. Biodiversity and its conservation in the Pantanal of Mato Grosso, Brazil. Aquat. Sci. 68, 278–309. https://doi.org/10.1007/s00027-006-0851-4

Junk, W.J., Nunes da Cunha, C., 2016. The Pantanal: A Brief Review of its Ecology, Biodiversity, and Protection Status, in: The Wetland Book. Springer Netherlands, Dordrecht, pp. 1–15. https://doi.org/10.1007/978-94-007-6173-5_129-1

Kattwinkel, M., Kühne, J.-V., Foit, K., Liess, M., 2011. Climate change, agricultural insecticide exposure, and risk for freshwater communities. Ecol. Appl. 21, 2068–2081. https://doi.org/10.1890/10-1993.1

Kimmel, C.B., Ballard, W.W., Kimmel, S.R., Ullmann, B., Schilling, T.F., 1995. Stages of Embryonic Development of the Zebrafish. Dev. Dyn. 203, 253–310. https://doi.org/203:255-310

Knie, J.L.W., Lopes, E.W.B., 2004. Testes Ecotoxicológicos: Métodos, técnicas e aplicações, FATMA/GTZ. ed.

Kumar, a., Correll, R., Grocke, S., Bajet, C., 2010. Toxicity of selected pesticides to freshwater shrimp, *Paratya australiensis* (Decapoda: Atyidae): Use of time series acute toxicity data to predict chronic lethality. Ecotoxicol. Environ. Saf. 73, 360–369. https://doi.org/10.1016/j.ecoenv.2009.09.001

Kwong, R.W.M., Kumai, Y., Perry, S.F., 2014. The physiology of fish at low pH : the zebrafish as a model system. Co. Biol. 651–662. https://doi.org/10.1242/jeb.091603

Laabs, V., Amelung, W., Pinto, A. a, Wantzen, M., da Silva, C.J., Zech, W., 2002. Pesticides in surface water, sediment, and rainfall of the northeastern Pantanal basin, Brazil. J. Environ. Qual. 31, 1636–1648. https://doi.org/10.2134/jeq2002.1636

Lammer, E., Carr, G.J., Wendler, K., Rawlings, J.M., Belanger, S.E., Braunbeck, T., 2009. Is the fish embryo toxicity test (FET) with the zebrafish (*Danio rerio*) a potential alternative for the fish acute toxicity test? Comp. Biochem. Physiol. Part C Toxicol. Pharmacol. 149, 196–209. https://doi.org/10.1016/J.CBPC.2008.11.006

Lorenzoni, I., Pidgeon, N.F., O'Connor, R.E., 2005. Dangerous Climate Change: The Role for Risk Research. Risk Anal. 25, 1387–1398. https://doi.org/10.1111/j.1539-6924.2005.00686.x

Maciel, C.R., Valenti, W.C., 2009. Biology, Fisheries, and Aquaculture of the Amazon River Prawn *Macrobrachium amazonicum*: A Review. Nauplius 17, 61–79.

Magalhães, C., 2003. Famílias *Pseudothdphusidae* e *Trichodactylidae*. In: Melo, G. A. S. (Ed) Manual de identificação dos Crustácea Decapoda de água doce do Brasil, Loyola. ed. São Paulo.

Maharajan, A., Narayanasamy, Y., Ganapiriya, V., Shanmugavel, K., 2015. Histological alterations of a combination of Chlorpyrifos and Cypermethrin (Nurocombi) insecticide in the fresh water crab, *Paratelphusa jacquemontii* (Rathbun). J. Basic Appl. Zool. 72, 104–112. https://doi.org/10.1016/j.jobaz.2015.08.002

Marengo, J.A., 2008. Water and Climate Change. Estud. avançados 22.

Marengo, J.A., Oliveira, G.S., Alves, L.M., 2015. Climate change scenarios in the Pantanal. In Dynamics of the Pantanal Wetland in South America, in: Springer Verlag. Springer, Cham, pp. 227–238. https://doi.org/10.1007/698_2015_357

Maria de Medeiros, V., Watanabe, T., Coler, R.R., Coler, R.A., 2001. Development of methods to assess the impact of herbicide use on the benthos of littoral impoundments in northeast Brazil. J. Aquat. Ecosyst. Stress Recover. 9, 67–71. https://doi.org/10.1023/A:1013199612093

Maund, S.J., Hamer, M.J., Lane, M.C.G., Farrelly, E., Rapley, J.H., Goggin, U.M., Gentle, W.E., 2002. Partitioning, bioavailability, and toxicity of the pyrethroid insecticide cypermethrin in sediments. Environ. Toxicol. Chem. 21, 9–15. https://doi.org/10.1002/etc.5620210102

Moe, S.J., De Schamphelaere, K., Clements, W.H., Sorensen, M.T., Van den Brink, P.J., Liess, M., 2013. Combined and interactive effects of global climate change and toxicants on populations and communities. Environ. Toxicol. Chem. 32, 49–61. https://doi.org/10.1002/etc.2045

Moraes-Riodades, P.M.C., Valenti, W.C., 2004. Morphotypes in male Amazon River Prawns, *Macrobrachium amazonicum*. Aquaculture 236, 297–307. https://doi.org/10.1016/j.aquaculture.2004.02.015

Moraes-Valenti, P., Valenti, W.C., 2010. Culture of the Amazon river prawn *Macrobrachium amazonicum*, in: New, M.B., Valenti, W.C., Tidwell, J.H. (Eds.), Freshwater Prawns: Biology and Farming. Wiley – Blackwell, EUA, pp. 485–497.

Moreira, J.C., Peres, F., Simões, A.C., Pignati, W.A., Dores, E.D.C., Vieira, S.N., Strüssmann, C., Mott, T., 2012. Contaminação de águas superficiais e de chuva por agrotóxicos em uma região do estado do Mato Grosso. Cien. Saude Colet. 17, 1557–1568. https://doi.org/10.1590/S1413-81232012000600019

Muniz, C.C., 2010. Avaliação do papel do pulso de inundação sobre a riqueza e biodiversidade de peixes em ambiente inundável, no sistema de baías caiçara, porção norte do Pantanal Matogrossense, alto Paraguai. Tese de doutorado. Universidade Federal de São Carlos.

Nadal, M., Marquès, M., Mari, M., Domingo, J.L., 2015. Climate change and environmental concentrations of POPs: A review. Environ. Res. 143, 177–185. https://doi.org/10.1016/j.envres.2015.10.012

New, P., Brown, A., Oliphant, A., Burchell, P., Smith, A., Thatje, S., 2014. The effects of temperature and pressure acclimation on the temperature and pressure tolerance of the shallow-water shrimp *Palaemonetes varians*. Mar. Biol. 161, 697–709. https://doi.org/10.1007/s00227-013-2371-9

Noyes, P.D., McElwee, M.K., Miller, H.D., Clark, B.W., Van Tiem, L.A., Walcott, K.C., Erwin, K.N., Levin, E.D., 2009. The toxicology of climate change: Environmental contaminants in a warming world. Environ. Int. 35, 971–986. https://doi.org/10.1016/j.envint.2009.02.006

NPTN, 1998. National Pesticide Information Center -Cypermethrin. Oregon.

Oliphant, A., Hauton, C., Thatje, S., 2013. The Implications of Temperature-Mediated Plasticity in Larval Instar Number for Development within a Marine Invertebrate, the Shrimp *Palaemonetes varians*. PLoS One 8, e75785. https://doi.org/10.1371/journal.pone.0075785

Oliphant, A., Thatje, S., 2014. Energetic adaptations to larval export within the brackish-water palaemonine shrimp, *Palaemonetes varians*. Mar. Ecol. Prog. Ser. 505, 177–191.

Oliphant, A., Thatje, S., Brown, A., Morini, M., Ravaux, J., Shillito, B., 2011. Pressure tolerance of the shallow-water caridean shrimp *Palaemonetes varians* across its thermal tolerance window. J. Exp. Biol. 214, 1109–1117. https://doi.org/10.1242/jeb.048058

Oliveira, E.F. de, Goulart, E., 2000. Distribuição espacial de peixes em ambientes lênticos : interação de fatores. Acta Sci. Biol. Sci. 22, 445–453. https://doi.org/10.4025/actascibiolsci.v22i0.2963

Oliveira, R. de, 2009. Zebrafish early life-stages and adults as a tool for ecotoxicity assessment. Dissertação de Mestrado, Universidade de Aveiro.

Oliveira, M.D., Hamilton, S.K., Calheiros, D.F., Jacobi, C.M., 2010. Oxygen Depletion Events Control the Invasive Golden Mussel (*Limnoperna fortunei*) in a Tropical Floodplain. Wetlands 30, 705–716. https://doi.org/10.1007/s13157-010-0081-3

Palma, J., Bureau, D.P., Andrade, J.P., 2008. Effects of binder type and binder addition on the growth of juvenile *Palaemonetes varians* and *Palaemon elegans* (Crustacea: *Palaemonidae*). Aquac. Int. 16, 427–436. https://doi.org/10.1007/s10499-007-9155-5

Palma, J., Bureau, D.P., Correia, M., Andrade, J.P., 2009. Effects of temperature, density and early weaning on the survival and growth of Atlantic ditch shrimp *Palaemonetes* *varians* larvae. Aquac. Res. 40, 1468–1473. https://doi.org/10.1111/j.1365-2109.2009.02245.x

Pavlaki, M.D., Araújo, M.J., Cardoso, D.N., Silva, A.R.R.S., Cruz, A., Mendo, S., Soares, M.V.M., Calado, R., Loureiro, S., 2016. Ecotoxicity and genotoxicity of cadmium in different marine trophic levels. Environ. Pollut. 215, 203–212. https://doi.org/10.1016/j.envpol.2016.05.010

Pereira, G., Elisa, M., Silva, S., Moraes, E.C., 2010. Impactos climáticos das áreas alagadas no Bioma Pantanal. Embrapa Informática Agropecuária/INPE 190–199.

Pott, A., Pott, V.J., 2004. Features and conservation of the Brazilian Pantanal wetland. Wetl. Ecol. Manag. 12, 547–552. https://doi.org/10.1007/s11273-005-1754-1

Rainbow, P.S., Smith, B.D., 2013. Accumulation and detoxification of copper and zinc by the decapod crustacean *Palaemonetes varians* from diets of field-contaminated polychaetes *Nereis diversicolor*. J. Exp. Mar. Bio. Ecol. 449, 312–320. https://doi.org/10.1016/j.jembe.2013.09.022

Raj Pandrangi, Michael Petras, Steve Ralph, and M.V., 1995. Alkaline Single Cell Gel (Comet) Assay and Genotoxicity Monitoring Using Bullheads and Carp. Environ. Mol. Mutagen. 26:345-356.

Ramsar, 2010. Cuidar das zonas úmidas - uma resposta às mudanças climáticas, Ramsar. Brasília - DF.

Revathi, P., Iyapparaj, P., Arockia Vasanthi, L., Munuswamy, N., Arun Prasanna, V., Pandiyarajan, J., Krishnan, M., 2014. Influence of Short Term Exposure of TBT on the Male Reproductive Activity in Freshwater Prawn *Macrobrachium rosenbergii* (De Man). Bull. Environ. Contam. Toxicol. 93, 446–451. https://doi.org/10.1007/s00128-014-1332-4

Rezende-Filho, A.T., Valles, V., Furian, S., Oliveira, C.M.S.C., Ouardi, J., Barbiero, L., 2015. Impacts of Lithological and Anthropogenic Factors Affecting Water Chemistry in the Upper Paraguay River Basin. J. Environ. Qual. 44, 1832. https://doi.org/10.2134/jeq2015.01.0019

Rodríguez, F., Barroso, F.J., Galindo, M.D., 1993. Estudio biométrico y morfológico de los huevos de *Palaemonetes varzans* leach de dos localidades del suroeste Español. Limnética 9, 67–72.

Ross, J., Sanches, L., 2006. Plano de conservação da bacia do alto Paraguai e o zoneamento ecológico-econômico para o Brasil. An. 1º Simpósio Geotecnologias no Pantanal, Campo Gd. Bras. 1, 11–15.

Ruppert, E., Barnes, R., 1993. Crustáceos, in: Zoologia Dos Invertebrados. Roca, pp. 659–777.

Sánchez, C., Delgado, G., 2003. Biodisponibilidad de metales en agua salobre (3UPS) y su efecto tóxico en el langostino *Macrobrachium rosenbergii*. retel - Rev. Toxicol. en línea 2–11.

Silva, D., 2004. Limnological characteristics of the water bodies of the Corutuba Nesting Site in Brazil's Pantanal. Acta Limnol. Bras 16, 359–368.

Silva, J. dos S.V. da, Abdon, M. de M., 1998. Delimitação do Pantanal Brasileiro e suas sub-regiões, Pesquisa Agropecuária Brasileira. Embrapa Informação Tecnológica.

Silva, M.H.S. da, Passos, M.M. Dos, Sakamoto, A.Y., 2013. As Lagoas Salitradas do Pantanal da Nhecolândia: um estudo da paisagem baseado no modelo GTP – Geossistema, Território e Paisagem. Confin. Rev. Fr. géographie/Revista Fr. Geogr. https://doi.org/10.4000/confins.8614

Soares, M.P., Jesus, F., Almeida, A.R., Zlabek, V., Grabic, R., Domingues, I., Hayd, L., 2017. Endemic shrimp *Macrobrachium pantanalense* as a test species to assess potential contamination by pesticides in Pantanal (Brazil). Chemosphere 168, 1082–1092. https://doi.org/10.1016/j.chemosphere.2016.10.100

Spence, R., Fatema, M.K., Reichard, M., Huq, K.A., Wahab, M.A., Ahmed, Z.F., Smith, C., 2006. The distribution and habitat preferences of the zebrafish in Bangladesh. J. Fish Biol. 69, 1435–1448. https://doi.org/10.1111/j.1095-8649.2006.01206.x

Spence, R., Gerlach, G., Lawrence, C., Smith, C., 2007. The behaviour and ecology of the zebrafish, *Danio rerio*. Biol. Rev. 83, 13–34. https://doi.org/10.1111/j.1469-185X.2007.00030.x

Vega-Perez, L.A., 1984. Desenvolvimento larval de *Macrobrachium heterochirus* (Wiegmann, 1836), *Macrobrachium amazonicum* (Heller, 1862) e *Macrobrachium brasiliense* (Heller, 1862) (Crustacea, Decapoda, Palaemonidae) em laboratório. Tese de doutorado, Universidade de São Paulo - Brasil.

Vercesi, K., Hayd, L., 2015. Avaliação do número de ovos em diferentes estágios de desenvolvimento embrionário de *Macrobrachium pantanalense*. Bol. do Inst. Pesca 41, 655–663.

Vinícius, A., Ferreira, L., José, E., Castro, T., Silva, M., Barbosa, A., Luzia, M., Martins, N., Paiva, M., Neto, D.A., Andrade, A., Filho, S., Maria, C., Sampaio, D.S., 2014. Toxicity of cryoprotectants agents in freshwater prawn embryos of *Macrobrachium amazonicum*. Cambridge Univ. - Zygote. https://doi.org/10.1017/S0967199414000458

Wang, X., Li, E., Xiong, Z., Chen, K., Yu, N., Du, Z., Chen, L., 2013. Low Salinity Decreases the Tolerance to Two Pesticides, Beta-cypermethrin and Acephate, of White-leg Shrimp, *Litopenaeus vannamei*. J. Aquac. Res. Dev. 04, 190. https://doi.org/10.4172/2155-9546.1000190

Williams, M.B., Dennis, L., Miyasaki, N., Barry, R., Powell, M., Watts, S., Smith, D., 2016. Effect of Dietary Protein Source and Quantity on the Growth and Body Composition of Juvenile *Danio rerio*. FASEB J. 30, 915–28.

Willink, W., Chernoff, B., Alonso, L.E., Montambault, J.R., Lourival, R., 2000. A Biological Assessment of the Aquatic Ecosystems of the Pantanal, Mato Grosso do Sul, Brasil. RAP Bulletin of Biological Assessment 18, Washington, DC.

WoRMS, 2018a. WoRMS - World Register of Marine Species - *Macrobrachium amazonicum* (Heller, 1862). WoRMS.

WoRMS, 2018b. *Palaemon varians* Leach, 1813. World Regist. Mar. Species.

WWF, 2015. Áreas prioritárias para conservação da biodiversidade no Cerrado e Pantanal, Brasília: WWF-Brasil.

Yancheva, V., Velcheva, I., Stoyanova, S., Georgieva, E., 2016. Histological biomarkers in fish as a tool in ecological risk assessment and monitoring programs: a review. Appl Ecol Env. Res 14, 47–75.

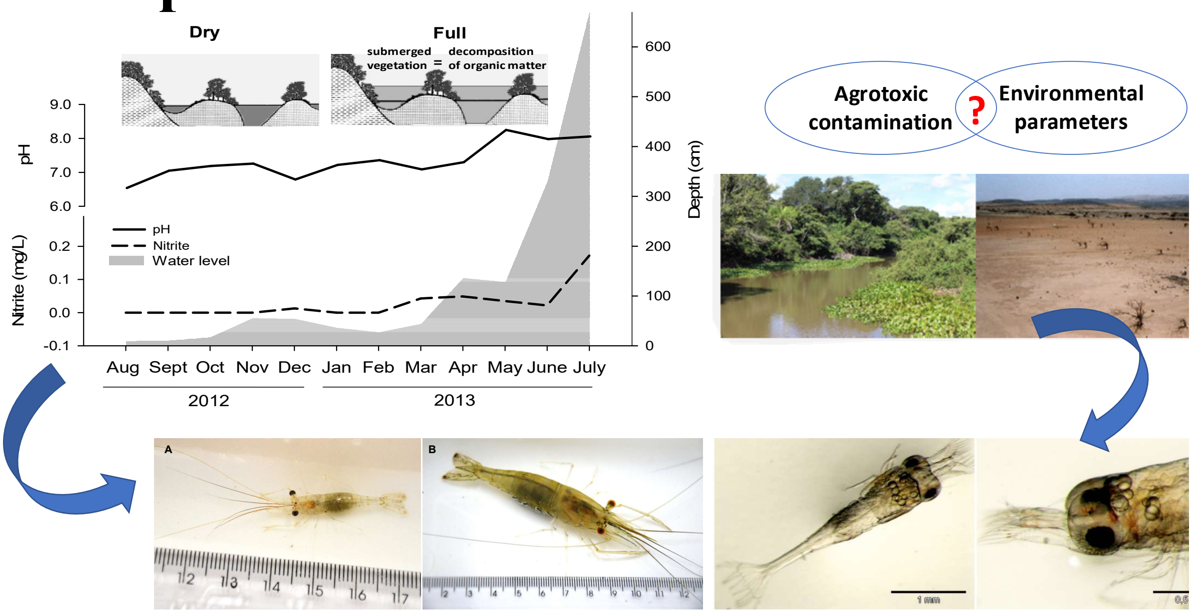
Zagatto, P.A., Bertoletti, E., 2008. Ecotoxicologia Aquática Princípios e Aplicações, 2nd ed. Rima, São Carlos.

Zeilhofer, P., Calheiros, D.F., de Oliveira, M.D., de Carvalho Dores, E.F.G., Lima, G.A.R., Fantin-Cruz, I., 2016. Temporal patterns of water quality in the Pantanal floodplain and its contributing Cerrado upland rivers: implications for the interpretation of freshwater integrity. Wetl. Ecol. Manag. 24, 697–716. https://doi.org/10.1007/s11273-016-9497-8

Zhang, Q., Cheng, J., Xin, Q., 2015. Effects of tetracycline on developmental toxicity and molecular responses in zebrafish (*Danio rerio*) embryos. Ecotoxicology 24, 707–719. https://doi.org/10.1007/s10646-015-1417-9

Chapter 2

Effects of pH and nitrites on the toxicity of Barrage® in two species of freshwater shrimps



Effects of pH and nitrites on the toxicity of a cypermetrin-based pesticide to shrimps

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Abstract

The Pantanal-Brazil is a wetland region characterized by seasonal flooding. Hydrological cycles in the region influence the water physico-chemical parameters, causing seasonal variations in pH and nitrites. In a context of climate change, these variations can be exacerbated, and potential interactions with environmental contaminants should be taken into consideration. Thus, the objective of this study was to evaluate the effects of varying pH and nitrite concentrations on the toxicity of the cypermethrin-based pesticide Barrage®. Larvae of the endemic shrimp *Macrobrachium pantanalense* and of the estuarine Amazonian congener *Macrobrachium amazonicum* were exposed to cypermethrin under several pH levels (6.5 to 8.5), as well as under several nitrite concentrations (0.1 to 0.4 mg/L). The pH had direct effects on mortality and development of both species. In *M. pantanalense*, the lethal effects of cypermethrin were more pronounced at low pH (96h-LC_{50} = 0.004 µg/L at pH 6.5, and 0.146 µg/L at pH 8.5). For *M. amazonicum,* an opposite response was observed, with increasing cypermethrin toxicity at high pH (96h-LC_{50} = 0.110 µg/L at pH 7.5 and 0.044 µg/L at pH 8.5). Variations in pH also seemed to modify the sublethal effects of cypermethrin on larval growth and development in *M. pantanalense*. Nitrite concentrations affected larval growth of both species, modifying also the effects of cypermethrin on the larval development of *M. amazonicum*. This work shows the importance of considering abiotic factors in a context of climate changes either due to possible direct effects on the physiology of organisms or due to interactions with existing contaminants, particularly in fragile biomes such as Pantanal.

Keywords: climate changes; water physico-chemical parameters; pesticide formulation; Barrage®; *Macrobrachium pantanalense; Macrobrachium amazonicum*

1. Introduction

Pantanal is a biome characterized by an extensive wetland area, 140000 km^2, located in western Brazil and Bolivia (Junk et al., 2006). This biome has well-defined annual water regimes with a "high water" season, in which flooding occurs on the plains, followed by a dry season, in which the water is limited to rivers, water channels, ponds and wetlands. This annual flood cycle varies according to region and climate fluctuations, such as rainfall (Alho, 2008; Alpizar et al., 2011; Junk et al., 2006; Pott and Pott, 2004). This flood cycle translates into a seasonal variation of the water physicochemical parameters, namely pH and nitrite concentration (Fig. S1, supplemental material). Indeed, several studies reported pH fluctuations throughout the hydrological cycle at numerous spots. For instance, in a pond in Miranda (Mato Grosso do Sul - MS, Brazil), annual variations between 6.5 and 8.2 were recorded (Júnior, 2013). The higher pH values were found during the high water season (Fig. S1) which was suggested to be due to the leaching of alkaline substances produced by fires occurring during the dry season (Júnior, 2013). Other studies report the opposite trend, with higher pH values during the dry season. For example, in the Paraguay River Basin, Corumbá - MS, Brazil, the pH ranged from 4.5 to 8.1 (Tuiuiú Bay) and from 5.4 to 7.3 (Bracinho, an arm of the Paraguay River, about 29 km long) during flood and dry seasons, respectively (Andrade, 2011). A similar response was verified in northern Pantanal (MT, Brazil) (Silva, 2004), where variations were attributed to the reduction of water level, increased evaporation, decomposition of macrophytes and presence of large numbers of birds in this region. Factors resulting from human activity, such as cattle farming and construction of small dams, may also influence pH variation (de Oliveira and Calheiros, 2011). The pH plays an important role in aquatic ecosystems as it directly interferes with the distribution and abundance of organisms (Araújo and Tejerina-Garro, 2009; Kemenes et al., 2010; Tondato et al., 2010). Its variation can reduce reproductive, respiratory, locomotor and feed efficiencies, besides altering several cellular and molecular mechanisms (Fromm, 1980; Kwong et al., 2014; Oliveira and Goulart, 2000).

The fluctuation of nitrite concentrations, in its turn, is related to the nitrogen cycle. During the high water season, decomposition of the submerged vegetation causes a drastic reduction of oxygen and release of ammonia due to the microbial decomposition of organic residues and nitrogenous excretions, a natural process known as "Decoada" (Calheiros et al., 2000; Hamilton et al., 1997, 1995; Oliveira et al., 2010; Zeilhofer et al., 2016). Ammonia undergoes a process of nitrification that is directly associated with variations of nitrite concentrations. In this sense, nitrite is relevant in the process of decomposition of organic matter when the vegetation is submerged in rivers, ponds and bays of Pantanal in the flood period (Silva, 2004). Thus, higher nitrite concentrations were observed during the high water season, reaching values as 170 µg/L (Júnior, 2013) and 64.4 µg/L (Rezende-Filho et al., 2015). Nitrites may interfere with physiological and metabolic processes that are vital to organisms (Jensen, 2003). Therefore, increases in nitrite concentrations in aquatic environments (of industrial, urban sewage and aquaculture or agriculture origin) are important due to their potential toxicity and/or interaction with other contaminants (Jensen, 2003).

Besides the cyclic variation of water physico-chemical properties, the aquatic systems in Pantanal also face an increased concentration of contaminants, namely pesticides. In fact, the notorious economic development in the agricultural and livestock farming that has been observed in Pantanal (Galdino et al., 2006) involves the use of pesticides to prevent and treat several pests, aiming to ensure good productivity. As a result, pesticide residues have reached the water bodies, compromising the environmental quality, biodiversity and the fragile equilibrium of the ecosystem (Alpizar et al., 2011; Galdino et al., 2006). One of the pesticides which has been found in surface and rainwater in Pantanal is cypermethrin, the active ingredient of the formulation Barrage®, which is widely used in Pantanal as a pesticide for agricultural applications, for domestic purposes (tick and insects in household residences) and for cattle farming to control ectoparasites (Barros, 1992; Gomes et al., 2011; NPTN, 1998). A previous study (Soares et al., 2017) showed that this pesticide is highly toxic to the endemic freshwater shrimp *Macrobrachium pantanalense* (96 h-LC50 = 0.05 µg/L). This raises concern about its potential effects under the seasonal flood cycle, i.e., under varying water physico-chemical parameters. Indeed, several studies showed the influence of pH variation on the toxicity of diverse compounds, namely aluminum (Dietrich and Schlatter, 1989), the herbicide formulation Vision® (Chen et al., 2004), the glyphosate-based herbicide formulation Roundup® (Tsui and Chu, 2003) and the pharmaceutical fluoxetine (Nakamura et al., 2008). Many of the effects are associated with changes in the rate of degradation, bioaccumulation or bioavailability of the compound (Dietrich and Schlatter, 1989; Maund et al., 2002). However, the effects of the variation of other parameters, namely nitrites, on the toxicity of contaminants are scarcely known.

From this point of view, special attention should be paid to climate change in this type of biome as it is responsible for increased fluctuations in water regime and, consequently, in the limnological parameters (Alho, 2008; Alpizar et al., 2011; Andrade et al., 2017). Therefore, climate change can lead to variations in greater extent or greater unpredictability in limnological parameters that are already intrinsically variable in this ecosystem. For instance, longer dry periods may decrease river dilution capacity, worsening water quality by concentrating nutrients and contaminants (Marengo, 2008; Pereira et al., 2010; Ramsar, 2010). Thus, expected variations in pH and nitrite concentration (Alpizar et al., 2011; Soer, 2010) may become a risk factor to aquatic organisms not so much due to exceeding limits of tolerance but mainly because of the possible interactions with chemical stressors. This highlights the importance of conducting studies on the risk assessment of pesticides to aquatic organisms, under varying water physico-chemical parameters. Thus, the objective of this study was to evaluate the influence of pH and nitrite variations on the toxicity of the cypermethrin-based pesticide Barrage® the effects were assessed in two shrimp species: *Macrobrachium pantanalense*, an endemic species of Pantanal (Dos Santos et al., 2013) and *Macrobrachium amazonicum*, its estuarine Amazonian congener, widely distributed in South America (Magalhães, 2003). The toxicity of cypermethrin to these organisms was evaluated at different pH levels and nitrite concentrations using mortality, growth and delays in larval development as endpoints. The pH levels (6.5, 7.5 and 8.5) and nitrite concentrations (0.1, 0.2 and 0.4 mg/L) were selected taking in consideration both the limits of tolerance of both species and also the environmental variation of these limnological parameters in the Pantanal water bodies.

2. Material and Methods

2.1 Chemicals

The formulation Barrage® (bought from Zoetis-Fort Dodge (Campinas, SP, Brazil)) is a concentrated emulsifiable suspension, with a cypermethrin concentration of 150 g/L. The exposure solutions were prepared by adding the chemical to culture medium (see section 2.2) adjusted for the desired pH or nitrite concentrations.

2.2 Test organisms

Parent shrimp of *Macrobrachium pantanalense* were collected in Lagoa Baiazinha (latitude: 20º15'49"S and longitude: 56º23'11"W), in Miranda, Pantanal of Mato Grosso do Sul (MS, Brazil), whereas parent shrimp of *Macrobrachium amazonicum* were provided by the Aquaculture Study Center of the São Paulo State University CAUNESP-Jaboticabal - (SP, Brazil). Parent shrimp of both species were kept in a closed, recirculating system, with a biofilter, according to the system developed by Calado et al., (2007). Culture medium was prepared using underground freshwater adjusted to conductivity of 0.24 µS/cm, pH of 7.5 ± 0.5, and dissolved oxygen above 8 mg/L. Animals were kept at a temperature of 28 ± 1 °C , a photoperiod cycle of 12 h:12 h (light:dark) and fed twice a day with adjusted diet (dry basis, containing 30% of crude protein and 4200 kcal/kg of gross energy) and fish fillet, following the common laboratory procedure. Ovigerous females in the final stages of embryo development were isolated in individual 25-L tanks with a plastic net, dividing them into two parts: one with shelters (pieces of PVC tube of 32 mm of diameter) that serve as a refuge for ovigerous females, and the other with a focused beam of LED light. Newly hatched larvae cross the net into the lighted part due to positive phototaxis, preventing cannibalism of the larvae. Newly hatched larvae were submitted to a disinfection bath with 0.025% of sodium hypochlorite to prevent infection with parasites and fungi and kept in culture medium similar to the described for adults but with salinity adjusted to 5 and 10 for *M. pantanalense*and *M. amazonicum*, respectively (salinity was adjusted with Nutratec salt, Nutratec, SP-Brazil). Culture medium was partly (70-80%) renewed every day. Larvae were kept in a chamber with controlled temperature and photoperiod (BOD Incubator, TE-401 model, Tecnal, Brazil) and fed daily with newly-hatched brine shrimp. Two-day old larvae were used in the assays.

2.3 Acute tests with shrimp larvae

The tests were performed in polyethylene 6-wells microplates with 5 replicates per treatment. Each replicate consisted of 5 larvae placed in 10 mL of solution per well.

Combinations of 2 factors were tested: the environmental parameter (pH or nitrite) and cypermethrin, following a completely randomized experimental design in a 3x6 factorial arrangement: 3 pH levels (6.5, 7.5 and 8.5) or 3 nitrite concentrations (0.1, 0.2 and 0.4 mg/L) were combined with 6 cypermethrin concentrations (0.00, 0.02, 0.04, 0.06, 0.18 and 0.55 μg/L).

2.3.1 Combination of pH x cypermethrin

Culture media at different pH levels were prepared using specific buffers (acquired from Sigma-Aldrich®) for pH stabilization. For pH 6.5, the buffer 2-(N-Morpholino) ethanesulfonic acid hydrate, 4-Morpholineethanesulfonic acid (MES, M2933) at 780 mg/L was used as buffer; for pH 7.5, 3-(N-Morpholino) propanesulfonic acid, 4-Morpholinepropanesulfonic acid (MOPS, M1254) at 837 mg/L was used; and for pH 8.5, 2-(Cyclohexylamino) ethanesulfonic acid (CHES, C2885) at 4560 mg/L was used. They were diluted in culture medium for larvae and pH adjusted with hydrochloric acid-HCl or sodium hydroxide-NaOH using a portable multi-parameter device (Combo pH/EC/TDS/ºC, J. ROMA, Lda.). Subsequently, the formulation Barrage® was added to pH-corrected culture media to attain the desired test concentrations of cypermethrin.

2.3.2 Combination of nitrite x cypermethrin

A stock solution of 1000 mg/L of nitrite was prepared by dissolving potassium nitrite (KNO_2) in distilled water. This solution was diluted in culture medium for larvae to attain the desired nitrite concentrations (0.1, 0.2 and 0.4 mg/L). Subsequently, Barrage® was added to nitrite-corrected culture media to attain the desired test concentrations of cypermethrin.

Nitrite concentrations were determined to confirm the prepared concentrations, following the procedure by Mackereth et al., (1978). Two 25 mL samples of each nitrite concentration were placed in test tubes; 0.5 mL of sulfanilamide solution (5 g diluted in 500 ml of distilled water) was added and, after 8 minutes, 0.5 mL of N-(1-naphthyl) ethylenediamine dichlorhydrate solution (0.5 g diluted in 500 ml of distilled water) was added and the samples were shaken; after 10 minutes, the absorbance was read at 543 nm against blank.

2.3.3 Test conditions

The duration of the exposure was 4 days. During this period, larvae were kept at 28 ± 1 °C under a photoperiod cycle of 12 h:12 h (light:dark). Larvae were daily checked for mortality, using a stereomicroscope. Test solutions were renewed daily after larvae feeding with newly hatched *Artemia*. At the end of the test larvae were anesthetized with carbonated water and fixed in 70% ethanol for further analysis. Larval development and carapace length were assessed using a stereomicroscope. The larval development was assessed by identifying the developmental stages (zoea) according to Vega-Perez (Vega-Perez, 1984). The carapace length (CL) was measured from the end of the rostrum (tip) to the median posterior edge of the carapace (Soares et al., 2017).

2.4 Statistical analyses

The calculation of the median lethal concentration values (LC_{50}; concentration that causes mortality in 50% of the organisms) was performed using the Probit analysis in the computer statistical software package Minitab 17 (Minitab 17 Statistical Software, 2010). Logistic regression models were built for mortality (binary variable) using cypermethrin concentration and pH/nitrite concentration as fixed factors. Linear regression models were built for growth data. To address the possible influence of environmental factors in cypermethrin toxicity (through the formulation Barrage®) the interaction term (cypermethrin x pH or cypermethrin x nitrite) was also included in every model. When the interaction term was not significant, the statistical analysis was repeated excluding this term. The analyses were performed using R 3.5.1 software. As the sequential development of larval stages were distinguished on an ordinal scale of increasing maturation, larval development data was transformed in terms of log-odds and analyzed with respect to cypermethrin concentration, and nitrite or pH by logistic regression using the function "polr" in the library MASS of R software (R Core Team, 2018).

3. Results

3.1 Combined effects of pH and cypermethrin

The combined effects of pH and cypermethrin on the survival of larvae of *M. pantanalense* and *M. amazonicum* are shown in Fig. S2 (response curves) and Table 1 (LC_{50} values). No interactive effects between pH and cypermethrin were observed; thus, statistical analysis without interaction was considered. For both species, pH and cypermethrin concentrations significantly affected mortality (see Table S1 for detailed statistics). For *M. pantanalense,* cypermethrin toxicity tended to decrease with pH (96h-LC_{50} = 0.004 µg/L at pH 6.5, and 0.146 µg/L at pH 8.5). For *M. amazonicum,* an opposite response was observed, as cypermethrin toxicity increased at pH 8.5 in comparison with the other tested levels (96h-LC_{50} = 0.110 µg/L at pH 7.5 and 0.044 µg/L at pH 8.5; Table 1, Fig. S2-B).

Table 1 - LC_{50} values, standard error (SE) and 95 % confidence interval for the toxicity of the cypermethrin-based formulation (Barrage®) to *Macrobrachium pantanalense* and *Macrobrachium amazonicum* at three different pH levels, after 4 days of exposure.

pH	LC_{50} (µg/L) ± SE	Confidence interval 95%	
		Lower limit	Upper limit
		M. pantanalense	
6.5	0.004 ± 0.012	-0.033	0.144
7.5	0.099 ± 0.015	0.073	0.140
8.5	0.146 ± 0.024	0.108	0.220
		M. amazonicum	
6.5	0.110 ± 0.013	0.088	0.144
7.5	0.131 ± 0.013	0.107	0.165
8.5	0.044 ± 0.005	0.034	0.060

The combined effects of pH and cypermethrin on the larval growth of both shrimp species are shown in Fig. 1 and Table S2. In *M. pantanalense*, the adverse effects of increasing cypermethrin concentration are more pronounced at low pH (6.5) than at high pH (8.5). This pattern of response is supported by the interaction verified between the two factors ($p = 8.52 \times 10^{-5}$). Concerning *M. amazonicum*, growth was affected by pH ($p = 6.85 \times 10^{-13}$) but not by cypermethrin ($p = 0.129$) and no interaction between the two factors was verified ($p = 0.485$).

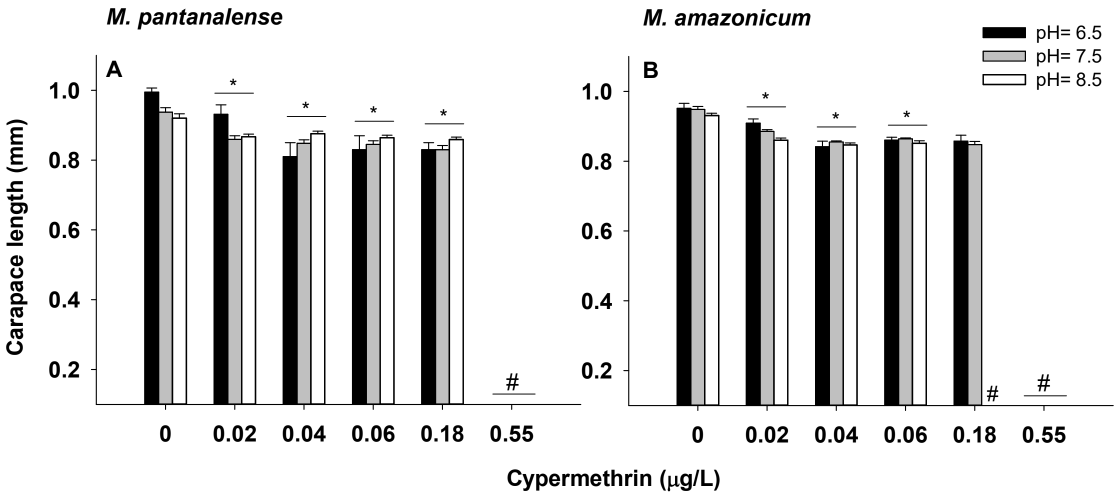

Figure 1. Cypermethrin effects (formulation Barrage®) at three pH levels (6.5, 7.5 and 8.5) on the growth of larvae of *M. pantanalense* (A) and *M. amazonicum* (B) after 4 days of exposure. Values represent means and the error bars represent standard errors. Asterisks denote statistically significant differences relative to the control for Barrage® ($p<0.05$, Dunnett's test). "#" indicates insufficient data to perform the analysis.

The combined effects of pH and cypermethrin on the larval development of both shrimp species are shown in Fig. 2 and Table S3. The development of larvae of *M. pantanalense* (Fig. 2 A-C) was delayed by cypermethrin ($p= 1.51x10^{-11}$): exposed larvae were mainly in zoea III or IV, whereas control larvae were mainly in zoea V. On the other hand, the deleterious effects of increasing cypermethrin concentration are more pronounced at high pH (8.5) than at low pH (6.5). This is supported by the significant interaction of both factors observed in the statistical analysis ($p= 1.95x10^{-4}$). Regarding *M. amazonicum*, their development was also delayed by cypermethrin ($p= 1.23x10^{-10}$). Moreover, increasing pH appears to delay larval development at pH 6.5 and 8.5 ($p= 2.32x10^{-7}$). However, no significant interaction of both factors was found ($p= 0.934$).

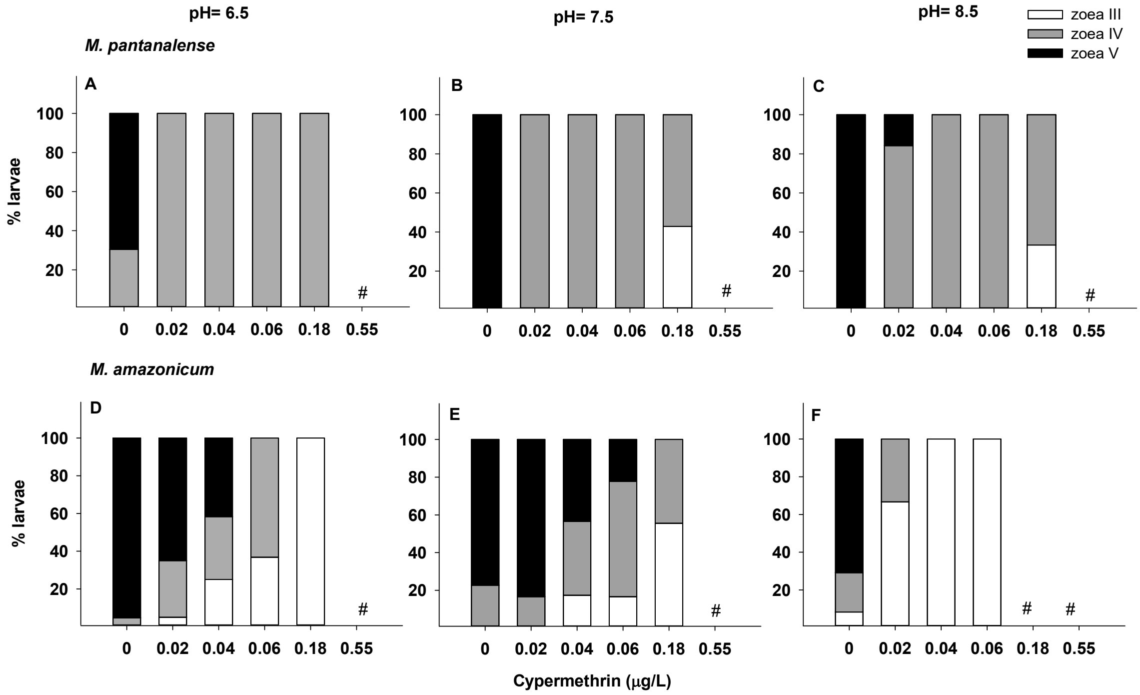

Figure 2. Cypermethrin effects (formulation Barrage®) at 3 pH levels (6.5, 7.5 and 8.5) on the developmental stage of larvae of *M. pantanalense* (A, B and C) and *M. amazonicum* (D, E and F) after 4 days of exposure. "#" indicates insufficient data to perform the analysis.

3.2 Combined effects of nitrite and cypermethrin

The measured nitrite concentrations confirmed the nominal concentrations. For 0.1, 0.2 and 0.4 mg/L of nitrite, the error was 4.84, 1.23 and -0.57%, respectively.

The combined effects of nitrite and cypermethrin on the survival of larvae of the shrimps *M. pantanalense* and *M. amazonicum* are shown in Fig. S3 (response curves) and Table 2 (LC_{50} values). There were no significant effects of nitrite concentrations in the mortality rates of *M. amazonicum* or *M. pantanalense* larvae ($p= 0.14$ and $p= 0.072$, respectively). Interaction between the two factors was neither observed ($p= 0.56$ and $p= 0.414$, respectively, Table S1). However, a significant effect of cypermethrin concentrations was found for both shrimp species ($p<2\times10^{-6}$ for both species, Table S1), with decreasing survival with increasing cypermethrin concentrations.

Table 2 - LC_{50} values, standard error (SE) and confidence interval for the toxicity of the cypermethrin-based formulation (Barrage®) to *Macrobrachium pantanalense* and *Macrobrachium amazonicum* at three different nitrite levels, after 4 days of exposure.

Nitrite (mg/L)	LC_{50} (µg/L) ± SE	Confidence interval 95%	
		Lower limit	Upper limit
		M. pantanalense	
0.1	0.025 ± 0.003	0.016	0.033
0.2	0.030 ± 0.006	0.015	0.043
0.4	0.019 ± 0.004	0.008	0.026
		M. amazonicum	
0.1	0.133 ± 0.020	0.100	0.194
0.2	0.125 ± 0.015	0.099	0.166
0.4	0.150 ± 0.022	0.115	0.216

The combined effects of nitrite and cypermethrin on the larval growth of both species are depicted in Fig. 3 and Table S2. Carapace length was significantly reduced by cypermethrin either in *M. pantanalense* ($p= 6.47x10^{-9}$) or *M. amazonicum* ($p= 3.84x10^{-15}$) (Fig. 3 A and B). Increasing nitrites seemed to reduce carapace length of *M. pantanalense* larvae ($p = 0.001$) and increase the carapace length of *M. amazonicum* larvae ($p= 2x10^{-16}$). For both species no interaction between the two factors was observed.

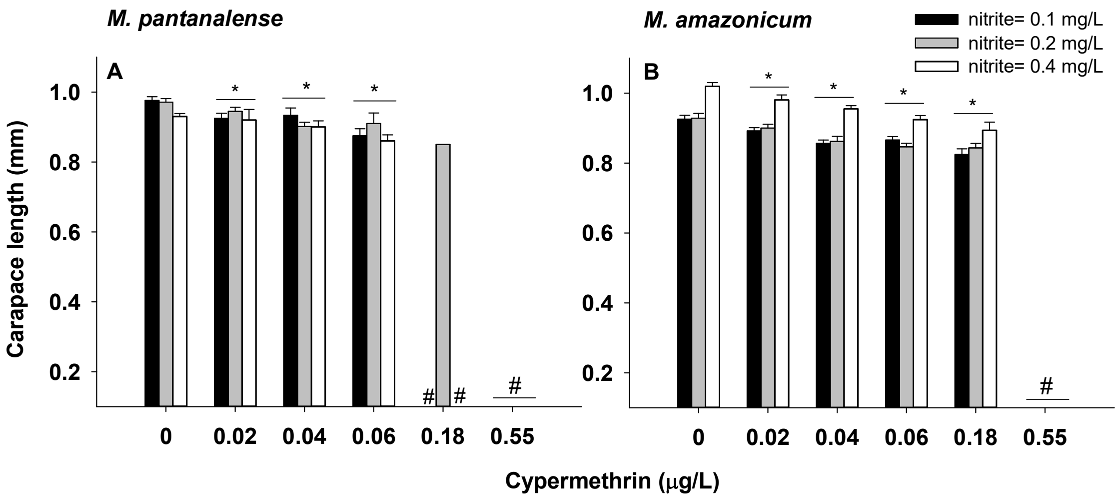

Figure 3. Cypermethrin effects (formulation Barrage®) in combination with different nitrite concentrations on the growth of larvae of *M. pantanalense* (A) and *M. amazonicum* (B) after 4 days of exposure. Values represent means and the error bars represent standard errors. Asterisks denote statistically significant differences relative to the control for Barrage® ($p<0.05$, Dunnett's test). "#" indicates insufficient data to perform the analysis.

The combined effects of nitrite and cypermethrin on the larval development of both shrimp species are presented in Fig. 4 and Table S3. The larval development of *M. pantanalense* was delayed by cypermethrin (p= $2.17x10^{-10}$): zoea V larvae were observed only in the control treatment, whereas zoea III larvae were found mainly at higher cypermethrin concentrations. No effects of nitrite concentrations (p= 0.33) or interaction between the two factors (p= 0.054) were observed. Concerning *M. amazonicum*, cypermethrin appears to delay their larval development (p= $2.19x10^{-16}$), whereas nitrite appears to boost it (p= $4.21x10^{-9}$). An interaction between the two factors (p=$4.92x10^{-6}$) was observed.

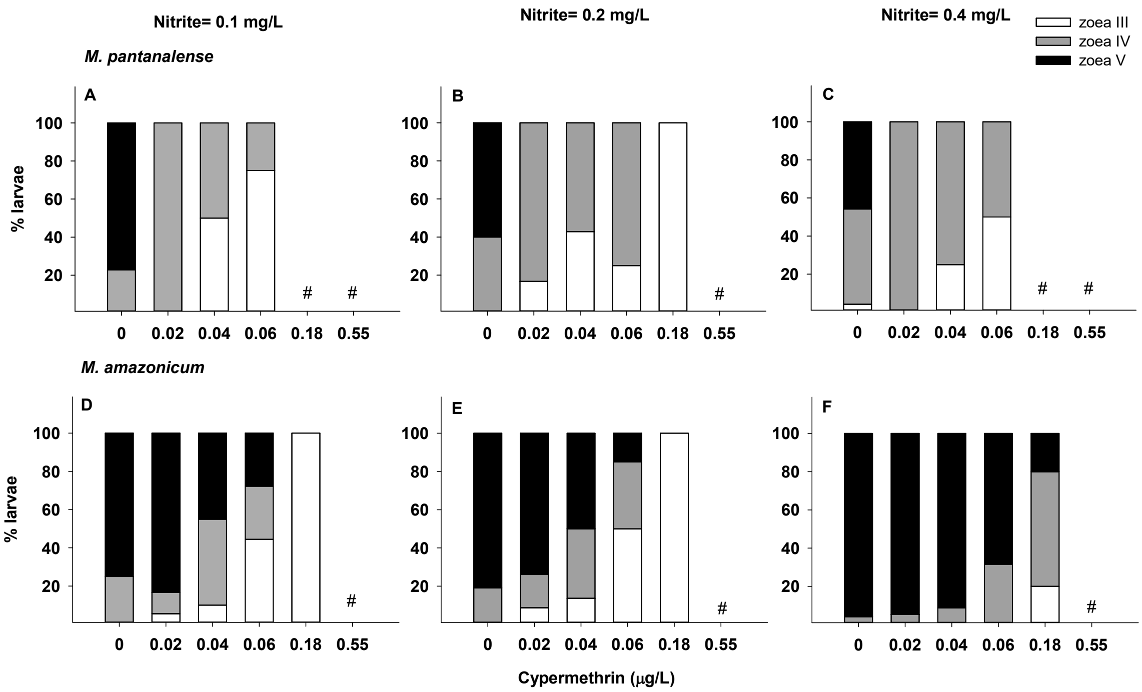

Figure 4. Cypermethrin effects (formulation Barrage®) in combination with different nitrite concentrations on the developmental stage of larvae of *M. pantanalense* (A, B and C) and *M. amazonicum* (D, E and F) after 4 d of exposure."#" indicates insufficient data to perform the analysis.

4. Discussion

In this work the combined effects of the cypermethrin-base Barrage® and two water physico-chemical parameters (pH or nitrite concentrations) were investigated using both lethal and sublethal endpoints in two Brazilian shrimp species. Main results showed that *M.*

pantanalense was more sensitive than *M. amazonicum* to the lethal effects of Barrage® under the tested environmental scenarios, as given by the lower LC50 values at all pH levels and nitrite concentrations. Also, pH showed to be very relevant, since it affected all the tested endpoints under all the tested environmental scenarios.

Combined effects of pH and cypermethrin

The LC_{50} values found for *M. amazonicum* and *M. pantanalense* at pH 7.5 agree with previous studies reporting LC_{50} values of 0.10 and 0.05 μg/L, respectively, for the same cypermethrin-based formulation (Soares et al., 2017). The results also suggest that the acute toxicity of the cypermethrin-based Barrage® formulation may be changed by pH although patterns of response differed between both shrimp species: the highest pH tested (8.5) decreased the toxicity of cypermethrin for *M. pantanalense* but increased it for *M. amazonicum*. The influence of pH on pesticide toxicity has been reported in several studies and it is usually associated with changes in hydrolysis rates, thereby altering compound availability to organisms (Chen et al., 2004). The hydrolysis rate of cypermethrin is pH-dependent: at pH 8.38 a 31.2% degradation was observed after 24h, whereas at pH 6.00, only a 8% degradation was observed (Al-Mughrabi et al., 1992); also, at neutral or acidic pH, the half-life of cypermethrin is greater than 50 days (Extoxnet, 1996; Kamrin, 1997). This pH-dependent degradation agrees with a previous study which reported that the efficiency of cypermethrin for the control of the tick parasite *Rhipicephalus microplus* was higher at lower pH (Garcia, 2007). This is in agreement with the results obtained for *M. pantanalense*: a decrease in cypermethrin toxicity at pH 8.5. The opposite result observed for *M. amazonicum* might be related to differential species-sensitivity to cypermethrin, their degradation metabolites, the inert agents of the formulation and/or their interaction. According to Xie et al (2008), the most persistent metabolite of cypermethrin is 3-phenoxybenzoic acid. This compound is also a degradation product of the pyrethroid permethrin and, according to Stratton and Corke (1982), it can be more toxic to algae and bacteria than the parent compound. In addition, the authors studied the interaction of metabolites with permethrin, reporting some synergistic effects. Attention must also be given to the inert agents present in the formulation Barrage® (C9 aromatic compounds and a minority of C8 and C10 aromatics) (Soares et al., 2017) which may also contribute to the toxicity of the formulation and have their toxicity modified by pH. Therefore, to a better understanding of the difference in the sensitivities of both shrimp to the intervening compounds, testing with the individualized components would have to be done.

At the sublethal level, cypermethrin decreased carapace length, as already reported in Soares et al. (2017) and other shrimp species (Collins and Cappello, 2006). pH variation itself did not affect growth although an interaction was detected between the two factors (pesticide and pH) in the case of *M. pantanalense*, suggesting an effect of pH in the toxicity of the pesticide. Overall, low pH levels have been associated with reductions of body size in several groups of organisms such as fish (larvae), bivalves, cladocerans and amphibians (Bechmann et al., 2011; Confer et al., 1983; Horne and Dunson, 1995; Lopes et al., 2001; Pierce, 1985). However, reductions of body size in shrimps are often found at pH levels lower than those tested in this study. This is the case of the study of Allan and Maguire (1992) with *Penaeus monodon*, where the reduction was only verified at a pH lower than 5.5.

A different tendency was observed for larval development, where clear effects of pH alone were detected for both species. In literature, there are evidences that pH reduction increases the larval development time, as observed in the shrimp *Pandalus borealis* at pH 7.6 relative to pH 8.1 (Arnberg et al., 2013; Bechmann et al., 2011). These effects may result from a compensation mechanism in which organisms use their energy reserves (lipid droplets) in response to chemical or environmental stress rather than towards larval development and growth (Hayd et al., 2014). In our study, increased larval development time was more pronouncedly observed for *M. amazonicum* by reducing pH from 7.5 to 6.5. The fact that larval development was faster at pH 6.5 for *M. pantanalense* and at pH 7.5 for *M. amazoniucum* might be related to the specific requirements of each species.

In this work, larvae of Pantanal and Amazon shrimps were responsive to variations in water pH, more specifically in the parameters mortality and larval development. Moreover, in the case of *M. pantanalense,* interaction between effects of pH and cypermethrin-based Barrage® on carapace length and larval development, resulting in unclear patterns or responses, suggests that pH may modify the toxicity of this compound and needs to be taken into account for accurate risk analysis. The type and nature of the interaction between these two factors should be further studied including its mechanism at a biochemical level.

Combined effects of Nitrite and cypermethrin

The tested nitrite concentrations did not affect cypermethrin lethal effects for neither of the species. At sublethal level, for *M. amazonicum* nitrites boosted growth and development of larvae. Moreover, interaction between nitrites and chemical for larval development was observed. The boost in larval development was also observed in juveniles of *Penaeus monodon* (Chen and Chen, 1992) but most of the studies available with crustaceans reported inhibition of growth and development associated with high nitrite concentrations (Gross et al., 2004; Hayd et al., 2014). A study with the same species (*M. amazonicum*) reported decrease in survival, productivity, mass gain and delay in the larval stage at concentrations above 0.8 mg/L (Hayd et al., 2014). Effects of nitrites may, thus, depend on the tested concentrations with pernicious effects being observed at higher levels than the tested in the present work. For *M. pantanalense*, nitrites also seemed to affect larval growth but, unlike for *M. amazonicum*, in this species nitrites inhibited growth accordingly to the reported in literature (Gross et al., 2004). Some mechanisms of action of nitrites reported in shrimp (*M. nipponense*) include altered immune response, such as imbalance in antioxidant defense and pro-oxidant action (Wang et al., 2004), accumulation in the hemolymph (in *Panaeus monodon* exposed to 2 mg/L) (Chen and Chen, 1992) and changes in hemocyanin, oxygen transport molecules in aquatic invertebrates, leading to hypoxia and death (Camargo and Alonso, 2006). The higher sensitivity of *M. pantanalense* to nitrites, compared to *M. amazonicum*, agrees with the higher sensitivity of this species to cypermethrin and copper (Soares et al., 2017). This highlights the importance of considering endemic species when assessing the risk of contaminants.

Results from this study suggest that oscillation of nitrite concentrations observed during the annual hydrological cycles of Pantanal may have a role on the development and growth of shrimp species, interfering with their life cycle. Moreover, it is unlikely that nitrites interfere with toxicity of cypermethrin at lethal level but modification of the toxicity of the compound at sublethal level cannot be excluded (as observed for *M. amazonicum* developmental stages).

The responses of both shrimp species to pH and nitrite variations are very similar, despite their biological requirements, particularly considering salinity. Indeed, Pantanal shrimp spend their entire life cycle in freshwater, as they inhabit inland waters (Santos et al., 2013). On the other hand, *M. amazonicum* have a territorial distribution throughout South America (Magalhães, 2003) and they develop in estuarine regions as they require an adequate salinity (10 ppt) for their larval development (Nóbrega et al., 2014).

The higher sensitivity of *M. pantanalense* than *M. amazonicum*, to the pesticide lethal effects under the tested environmental scenarios highlights the importance of considering endemic species when assessing the toxicity of environmental contaminants. Also, the use of a commercial formulation instead of the pure pesticide is ecologically relevant as, this way, the toxicity of all the formulation components and their interactions is taken into account, which represents a more realistic scenario.

Given that pH values are lower during the dry season (Júnior, 2013), we may state that *M. pantanalense* will be more sensitive to the pesticide during dry season than "high water" season, i.e., dry water season represents a very sensitive period for this endemic species. This, allied to the high sensitivity of *M. pantanalense* to cypermethrin, suggests that special attention to variation of the population of this species should be done during the dry season. In opposition, *M. amazonicum* was more sensitive to cypermethrin under high pH and, thus, will be more susceptible to the lethal effects of cypermethrin during the "high water" season. Such variability in the period of higher sensitivity to cypermethrin should be considered in further studies. Monitoring the environmental concentrations of cypermethrin and other highly toxic contaminants is also recommended. In Pantanal, cypermethrin residues have been found in rainwater in concentrations up to 0.52 mg/L (Moreira et al., 2012), as well as in surface waters and sediments (Calheiros et al., 2006). Such high concentrations, allied its high toxicity, particularly under specific water physico-chemical conditions (e.g. high pH for *M. amazonicum* and low pH for *M. pantanalense*) highlight the need to control pesticides use in this region in order to preserve the biodiversity typical of this biome.

Globally, this work shows the importance of considering the variation of water physico-chemical parameters when assessing the toxicity of contaminants to aquatic organisms and corroborates the relevant role of pH in the mortality, growth and development of both shrimp species. Both pH and nitrite affected the toxicity of the cypermethrin-based formulation Barrage®, but only in a few environmental scenarios.

However, it is relevant to conduct such assessment with other contaminants and/or other water physico-chemical parameters both because an increased use of pesticides and because a wider range of water physico-chemical parameters will occur as a consequence of climate changes. Indeed, if toxicity tests are carried out only under "optimal conditions", toxicity might be underestimated. For instance, the Barrage® toxicity to *M. pantanalense* at pH 6.5 (0.004 mg/L) was 25-fold higher than at pH 7.5 (0.099 mg/L; note that this pH level was used in a previous study for toxicity assessment with this species (Soares et al., 2017). The underestimation of Barrage® toxicity at low pH might be valid for other contaminants, which is a major concern, especially in fragile biomes as Pantanal. Thus, we suggest that water physico-chemical parameters should be taken into consideration in future studies for a more realistic risk assessment.

5. Conclusion

The tested pH levels, which are plausible to occur along the hydrological cycle in the Pantanal region, showed to have direct effects in the mortality and development of both test species. Moreover, in the case of *M. pantanalense,* pH seemed to modify the sublehtal effects of cypermethrin (growth and larval development). Thus, pH was shown to play a critical role in the life-cycle of these shrimps. Nitrite concentrations affected the larval growth of both tested species and also influenced cypermethrin effects on the larval developmental in *M. amazonicum*, but their effects were less pronounced than those of pH. This work showed the importance of considering abiotic factors in a context of climate changes either due to possible direct effects in the physiology of organisms as due to interaction with existing contaminants. Indeed, toxicity of pesticides might be underestimated if their assessment does not take into account the variation of water physico-chemical parameters.

The period (dry season or "high water" season) during which each species was most sensitive to cypermethrin differed for both species, which emphasizes the need to monitor and control the environmental concentrations of cypermethrin and other pesticides throughout the year, in order to contribute to the preservation of the biodiversity in Pantanal.

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6. References

Al-Mughrabi, K.I., Nazer, I.K., Al-Shuraiqi, Y.T., 1992. Effect of pH of water from the King AbdaHah Canal in Jordan on the stability of cypermethrin. Crop Prot. 11, 341–344. https://doi.org/0261-219419210410341-04

Alho, C.J.R., 2008. Biodiversity of the Pantanal: response to seasonal flooding regime and to environmental degradation. Brazilian J. Biol. 68, 957–966. https://doi.org/10.1590/S1519-69842008000500005

Alpizar, F., Carlsson, F., Naranjo, M.A., 2011. The effect of ambiguous risk, and coordination on farmers' adaptation to climate change — A framed field experiment. Ecol. Econ. 70, 2317–2326. https://doi.org/10.1016/j.ecolecon.2011.07.004

Andrade, M., 2011. O fenômeno da decoada no Pantanal do rio Paraguai, Corumbá/MS: alterações dos parâmetros limnológicos e efeitos sobre os macroinvertebrados bentônicos. Doctoral dissertation, Universidade de São Paulo.

Andrade, T.S., Henriques, J.F., Almeida, A.R., Soares, A.M.V.M., Scholz, S., Domingues, I., 2017. Zebrafish embryo tolerance to environmental stress factors—Concentration–dose response analysis of oxygen limitation, pH, and UV-light irradiation. Environ. Toxicol. Chem. 36, 682–690. https://doi.org/10.1002/etc.3579

Araújo, N.B., Tejerina-Garro, F.L., 2009. Influence of environmental variables and anthropogenic perturbations on stream fish assemblages, Upper Paraná River, Central

Brazil. Neotrop. Ichthyol. 7, 31–38. https://doi.org/10.1590/S1679-62252009000100005

Arnberg, M., Calosi, P., Spicer, J.I., Tandberg, A.H.S., Nilsen, M., Westerlund, S., Bechmann, R.K., 2013. Elevated temperature elicits greater effects than decreased pH on the development, feeding and metabolism of northern shrimp (*Pandalus borealis*) larvae. Mar. Biol. 160, 2037–2048. https://doi.org/10.1007/s00227-012-2072-9

Barros, A. de, 1992. Recomendações para controle da mosca-dos-chifres no Pantanal. Embrapa, Centro de Pesquisa Agropecuária do Pantanal (Corumba, MS). ainfo.cnptia.embrapa.br.

Bechmann, R.K., Taban, I.C., Westerlund, S., Godal, B.F., Arnberg, M., Vingen, S., Ingvarsdottir, A., Baussant, T., 2011. Effects of Ocean Acidification on Early Life Stages of Shrimp (*Pandalus borealis*) and Mussel (*Mytilus edulis*). J. Toxicol. Environ. Heal. Part A 74, 424–438. https://doi.org/10.1080/15287394.2011.550460

Calado, R., Vitorino, A., Dionísio, G., Dinis, M.T., 2007. A recirculated maturation system for marine ornamental decapods. Aquaculture 263, 68–74. https://doi.org/10.1016/j.aquaculture.2006.10.013

Calheiros, D.F., Oliveira, M.D., Dolores, E.F.G., 2006. Poluiçãoo por pesticidas, nutrientes e material em suspensão nos rios formadores do Pantanal Matogrossense Embrapa Pantanal (Corumbá, MS) p. 4. URL: https://www.infoteca.cnptia.embrapa.br/infoteca/bitstream/doc/812632/1/ADM096.pdf . AccessedJaneiro 2019.

Calheiros, D.F., Seidl, A.F., Ferreira, C.J., 2000. Participatory research methods in environmental science: local and scientific knowledge of a limnological phenomenon in the Pantanal wetland of Brazil. J. Appl. Ecol. 37, 684–696.

Camargo, J.A., Alonso, Á., 2006. Ecological and toxicological effects of inorganic nitrogen pollution in aquatic ecosystems: A global assessment. Environ. Int. 32, 831–849. https://doi.org/10.1016/j.envint.2006.05.002

Chen, C.Y., Hathaway, K.M., Folt, C.L., 2004. Multiple stress effects of Vision® herbicide, ph, and food on zooplankton and larval amphibian species from forest wetlands. Environ. Toxicol. Chem. 23, 823. https://doi.org/10.1897/03-108

Chen, J.-C., Chen, S.-F., 1992a. Effects of nitrite on growth and molting of *Penaeus* *monodon* juveniles. Comp. Biochem. Physiol. Part C Comp. Pharmacol. 101, 453–458. https://doi.org/10.1016/0742-8413(92)90069-J

Chen, J.-C., Chen, S.-F., 1992b. Accumulation of nitrite in the haemolymph of *Penaeus monodon* exposed to ambient nitrite. Comp. Biochem. Physiol. Part C Comp. Pharmacol. 103, 477–481. https://doi.org/10.1016/0742-8413(92)90168-7

Collins, P., Cappello, S., 2006. Cypermethrin Toxicity to Aquatic Life: Bioassays for the Freshwater Prawn *Palaemonetes argentinus*. Arch. Environ. Contam. Toxicol. 51, 79–85. https://doi.org/10.1007/s00244-005-0072-1

Confer, J.L., Kaaret, T., Likens, G.E., 1983. Zooplankton Diversity and Biomass in Recently Acidified Lakes. Can. J. Fish. Aquat. Sci. 40, 36–42. https://doi.org/10.1139/f83-006

de Oliveira, M.D., Calheiros, D.F., 2011. Qualidade da Água em Agroecossistemas do Pantanal: Sub-regiões da Nhecolândia e Poconé. Embrapa Pantanal-Boletim Pesqui. e Desenvolv. BP109.

Dietrich, D., Schlatter, C., 1989. Aluminium toxicity to rainbow trout at low pH. Aquat. Toxicol. 15, 197–212. https://doi.org/10.1016/0166-445X(89)90036-2

Dos Santos, A., Hayd, L., Anger, K., 2013. A new species of *Macrobrachium* Spence Bate, 1868 (Decapoda, Palaemonidae), *M. pantanalense*, from the Pantanal, Brazil. Zootaxa 3700, 534–546. https://doi.org/http://dx.doi.org/10.11646/zootaxa.3700.4.2

Extoxnet, 1996. Pesticide Information Profiles - Cypermethrin. URL http://extoxnet.orst.edu/pips/cypermet.htm (accessed 4.4.18).

Fromm, P. 0., 1980. A review of some physiological and toxicological responses of freshwater fish to acid stress. Env. BioL Fish 5, 79–93.

Galdino, S., Vieira, L.M., Pellegrin, L.A., 2006. Impactos Ambientais e Socioeconômicos na Bacia do Rio Taquari - Pantanal, 1º. ed. Embrapa Pantana, Corumbá.

Garcia, F., Lúcio, N., 2007. Influência do ph do diluidor , na ação de caldas ixodicidas (Amitraz , Clorpirifós e Cipermetrina), contra *Rhipicephalus (Boophilus) microplus* (Canestrini , 1887) (Acarina : Ixodidae). Faculdade de Ciências Agrárias e Veterinárias – UNESP.

Gomes, A., Koller, W., Barros, A., 2011. Susceptibility of *Rhipicephalus (Boophilus) microplus* to acaricides in Mato Grosso do Sul, Brazil. Ciência Rural 41, 1447–1452.

https://doi.org/http://dx.doi.org/10.1590/S0103-84782011005000105

Gross, A., Abutbul, S., Zilberg, D., 2004. Acute and Chronic Effects of Nitrite on White Shrimp , *Litopenaeus vannamei* , Cultured in Low-Salinity Brackish Water. J. World Aquac. Soc. 35, 315–321.

Hamilton, S.K., Sippel, S.J., Calheiros, bora F., Melack, J.M., 1997. An anoxic event and other biogeochemical effects of the Pantanal wetland on the Paraguay River. Limnol. Ocean. 42, 257–272.

Hamilton, S.K., Sippel, S.J., Melack, J.M., 1995. Oxygen depletion and carbon dioxide and methane production in waters of the Pantanal wetland of Brazil. Biogeochemistry 30, 115–141. https://doi.org/10.1007/BF00002727

Hayd, L. a., Lemos, D., Valenti, W.C., 2014. Effects of Ambient Nitrite on Amazon River Prawn, Macrobrachium amazonicum , larvae. J. World Aquac. Soc. 45, 55–64. https://doi.org/10.1111/jwas.12071

Horne, M.T., Dunson, W.A., 1995. The interactive effects of low pH, toxic metals, and DOC on a simulated temporary pond community. Environ. Pollut. 89, 155–161. https://doi.org/10.1016/0269-7491(94)00057-K

Jensen, F.B., 2003. Nitrite disrupts multiple physiological functions in aquatic animals. Comp. Biochem. Physiol. Part A Mol. Integr. Physiol. 135, 9–24. https://doi.org/10.1016/S1095-6433(02)00323-9

Júnior, R.C.M., 2013. Avaliação dos parâmetros físicos e químicos da lagoa Baiazinha, Pantanal de Miranda-MS. Dissertação de mestrado. Universidade Estadual do Mato Grosso do Sul.

Junk, W.J., da Cunha, C.N., Wantzen, K.M., Petermann, P., Strüssmann, C., Marques, M.I., Adis, J., 2006. Biodiversity and its conservation in the Pantanal of Mato Grosso, Brazil. Aquat. Sci. 68, 278–309. https://doi.org/10.1007/s00027-006-0851-4

Kamrin, M.A., 1997. Pesticide Profiles: Toxicity, Environmental Impact, and Fate, CRC press.

Kemenes, A., Rider Forsberg, B., Magalhães, C., Hélio, &, Anjos, D., 2010. Environmental factors influencing the community structure of shrimps and crabs (Crustacea: Decapoda) in headwater streams of the Rio Jaú, Central Amazon, Brazil, Pan-American Journal of Aquatic Sciences.

Kwong, R.W.M., Kumai, Y., Perry, S.F., 2014. The physiology of fish at low pH : the zebrafish as a model system. Co. Biol. 651–662. https://doi.org/10.1242/jeb.091603

Lopes, J.M., Silva, L.V.F., Baldisserotto, B., 2001. Survival and growth of silver catfish larvae exposed to different water pH. Aquac. Int. 9, 73–80. https://doi.org/10.1023/A:1012512211898

Mackereth, F.J.H., Heron, J., Talling, J.F., 1978. Water analysis: some revised methods for limnologists. Freshwater Biological

Association. 121p.

Magalhães, C., 2003. Famílias *Pseudothdphusidae* e *Trichodactylidae*. In: Melo, G. A. S. (Ed) Manual de identificação dos Crustácea Decapoda de água doce do Brasil, Loyola. ed. São Paulo.

Marengo, J.A., 2008. Water and Climate Change. Estud. Avançados 22, 83–96. https://doi.org/http://dx.doi.org/10.1590/S0103-40142008000200006

Maund, S.J., Hamer, M.J., Lane, M.C.G., Farrelly, E., Rapley, J.H., Goggin, U.M., Gentle, W.E., 2002. Partitioning, bioavailability, and toxicity of the pyrethroid insecticide cypermethrin in sediments. Environ. Toxicol. Chem. 21, 9–15. https://doi.org/10.1002/etc.5620210102

Minitab 14 Statistical Software, 2010. https://doi.org/[Computer software]. State College, PA: Minitab, Inc. (www.minitab.com)

Moreira, J.C., Peres, F., Simões, A.C., Pignati, W.A., Dores, E.D.C., Vieira, S.N., Strüssmann, C., Mott, T., 2012. Contaminação de águas superficiais e de chuva por agrotóxicos em uma região do estado do Mato Grosso. Cien. Saude Colet. 17, 1557–1568. https://doi.org/10.1590/S1413-81232012000600019

Nakamura, Y., Yamamoto, H., Sekizawa, J., Kondo, T., Hirai, N., Tatarazako, N., 2008. The effects of pH on fluoxetine in *Japanese medaka* (*Oryzias latipes*): Acute toxicity in fish larvae and bioaccumulation in juvenile fish. Chemosphere 70, 865–873. https://doi.org/10.1016/j.chemosphere.2007.06.089

Nóbrega, P.S.V. da, Bentes, B., Martinelli-Lemos, J.M., 2014. Population structure and relative growth of the Amazon shrimp *Macrobrachium amazonicum* (Heller, 1862) (Decapoda: Palaemonidae) on two islands in the fluvial-estuarine plain of the Brazilian Amazon. Nauplius 22, 13–20. https://doi.org/10.1590/S0104-

64972014000100002

NPTN, 1998. National Pesticide Information Center -Cypermethrin. Oregon.

Oliveira, E.F. de, Goulart, E., 2000. Distribuição espacial de peixes em ambientes lênticos : interação de fatores. Acta Sci. Biol. Sci. 22, 445–453. https://doi.org/10.4025/actascibiolsci.v22i0.2963

Oliveira, M.D., Hamilton, S.K., Calheiros, D.F., Jacobi, C.M., 2010. Oxygen Depletion Events Control the Invasive Golden Mussel (*Limnoperna fortunei*) in a Tropical Floodplain. Wetlands 30, 705–716. https://doi.org/10.1007/s13157-010-0081-3

Pereira, G., Elisa, M., Silva, S., Moraes, E.C., 2010. Impactos climáticos das áreas alagadas no Bioma Pantanal. Embrapa Informática Agropecuária/INPE 190–199.

Pierce, B.A., 1985. Acid Tolerance in Amphibians. Bioscience 35, 239–243. https://doi.org/10.2307/1310132

Pott, A., Pott, V.J., 2004. Features and conservation of the Brazilian Pantanal wetland. Wetl. Ecol. Manag. 12, 547–552. https://doi.org/10.1007/s11273-005-1754-1

R Core Team, 2018. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL http//www.R-project.org/.

Ramsar, 2010. Cuidar das zonas úmidas - uma resposta às mudanças climáticas, Ramsar. Brasília - DF.

Rezende-Filho, A.T., Valles, V., Furian, S., Oliveira, C.M.S.C., Ouardi, J., Barbiero, L., 2015. Impacts of Lithological and Anthropogenic Factors Affecting Water Chemistry in the Upper Paraguay River Basin. J. Environ. Qual. 44, 1832. https://doi.org/10.2134/jeq2015.01.0019

Silva, D., 2004. Limnological characteristics of the water bodies of the Corutuba Nesting Site in Brazil's Pantanal. Acta Limnol. Bras 16, 359–368.

Soares, M.P., Jesus, F., Almeida, A.R., Zlabek, V., Grabic, R., Domingues, I., Hayd, L., 2017. Endemic shrimp *Macrobrachium pantanalense* as a test species to assess potential contamination by pesticides in Pantanal (Brazil). Chemosphere 168, 1082–1092. https://doi.org/10.1016/j.chemosphere.2016.10.100

Soer, E., 2010. Understanding Climate Change. Eur. Environ. — state outlook.

Stratton, G.W., Corke, C.T., 1982. Toxicity of the insecticide permethrin and some degradation products towards algae and cyanobacteria. Environ. Pollut. Ser. A, Ecol. Biol. 29, 71–80. https://doi.org/10.1016/0143-1471(82)90055-1

Tondato, K.K., Mateus, L.A. de F., Ziober, S.R., 2010. Spatial and temporal distribution of fish larvae in marginal lagoons of Pantanal, Mato Grosso State, Brazil. Neotrop. Ichthyol. 8, 123–134. https://doi.org/10.1590/S1679-62252010005000002

Tsui, M.T.K., Chu, L.M., 2003. Aquatic toxicity of glyphosate-based formulations: comparison between different organisms and the effects of environmental factors. Chemosphere 52, 1189–1197. https://doi.org/10.1016/S0045-6535(03)00306-0

Vega-Perez, L.A., 1984. Desenvolvimento larval de *Macrobrachium heterochirus* (Wiegmann, 1836), *Macrobrachium amazonicum* (Heller, 1862) e *Macrobrachium brasiliense* (Heller, 1862) (Crustacea, Decapoda, Palaemonidae) em laboratório. Tese de doutorado, Universidade de São Paulo - Brasil.

Xie, W.-J., Zhou, J.-M., Wang, H.-Y., Chen, X.-Q., 2008. Effect of Nitrogen on the Degradation of Cypermethrin and Its Metabolite 3-Phenoxybenzoic Acid in Soil. Pedosphere 18, 638–644. https://doi.org/10.1016/S1002-0160(08)60058-2

Zeilhofer, P., Calheiros, D.F., de Oliveira, M.D., de Carvalho Dores, E.F.G., Lima, G.A.R., Fantin-Cruz, I., 2016. Temporal patterns of water quality in the Pantanal floodplain and its contributing Cerrado upland rivers: implications for the interpretation of freshwater integrity. Wetl. Ecol. Manag. 24, 697–716. https://doi.org/10.1007/s11273-016-9497-8

Supplementary data

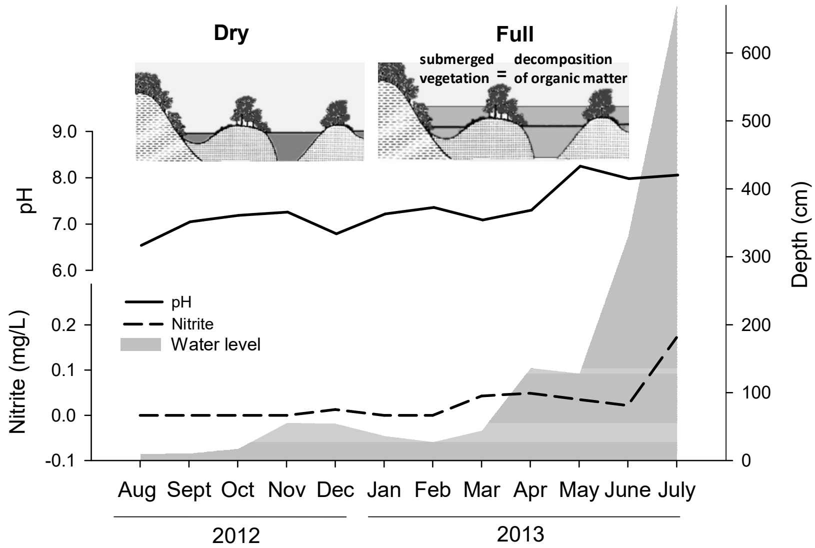


Figure S1. Schematic changes in pH levels and nitrite concentrations influenced by flood pulse in a Pantanal pond in Miranda, Mato Grosso do Sul (MS, Brazil) (adapted from Júnior, 2013).

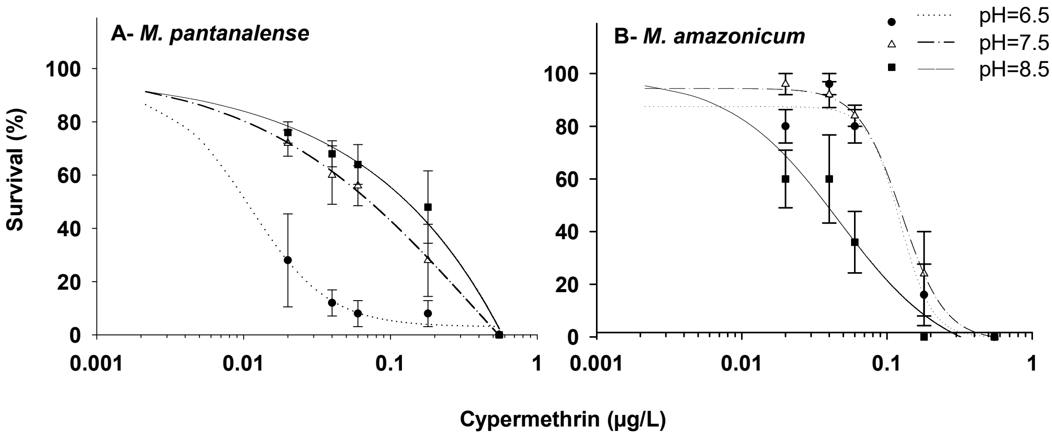


Figure S2. Cypermethrin effects (formulation Barrage®) on survival of larvae of *M. pantanalense* (A) and *M. amazonicum* (B) at 3 pH levels (6.5, 7.5 and 8.5). Symbols represent means and the error bars represent standard errors. Four-parameter log-logistic functions were used to fit data.

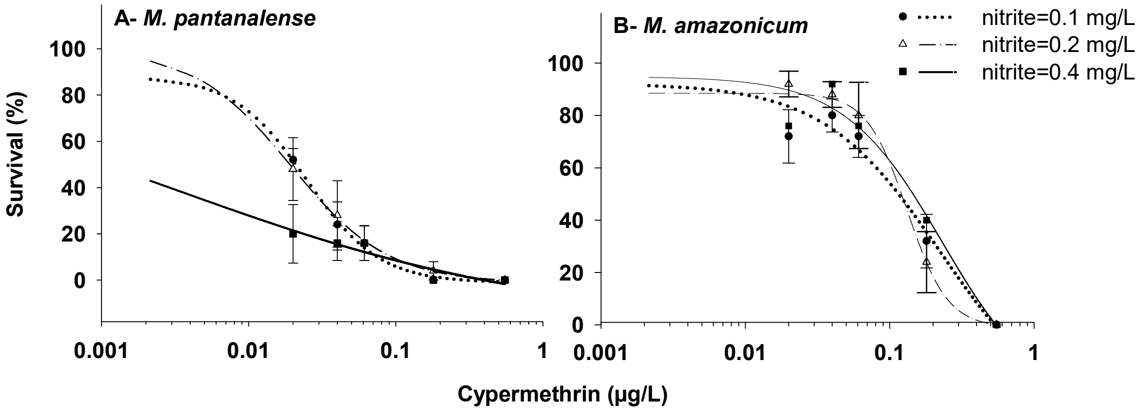


Figure S3. Cypermethrin effects (formulation Barrage®) in the survival of larvae of *M. pantanalense* (A) and *M. amazonicum* (B) at different nitrite levels (0.1, 0.2 and 0.4 mg/L). Symbols represent means and the error bars represent standard errors. Four-parameter log-logistic functions were used to fit data.

Table S1: Results of the logistic regression analysis (pH or nitrite x cypermethrin- based Barrage®) of shrimp mortality. Depending on the statistical significance of the interaction between both factors, the statistical analysis that best applies to each test is highlighted in grey. Statistically significant values are highlighted in bold.

	Model with interaction	Estimate	Std. Error	Z value	P value	No interaction model	Estimate	Std. Error	Z value	P value
pH x cypermethrin	**(Intercept)**	5.0893	1.5391	3.307	0.000944	**(Intercept)**	6.9287	1.1556	5.996	2.03E-09
	Barrage	54.5571	23.6904	2.303	**0.021283**	**Barrage**	15.1741	2.0934	7.249	**4.21E-13**
	pH	-0.8317	0.2055	-4.048	**5.18E-05**	**pH**	-1.0724	0.1587	-6.755	**1.42E-11**
M. pantanalense	**Interaction**	-4.9886	2.9399	-1.697	0.089721					
pH x cypermethrin	**(Intercept)**	-6.7494	2.0096	-3.359	0.000783	**(Intercept)**	-7.9317	1.4473	-5.48	4.25E-08
	Barrage	4.0164	24.3824	0.165	0.869161	**Barrage**	24.0049	2.5952	9.25	**< 2E-16**
	pH	0.5961	0.2608	2.286	**0.022256**	**pH**	0.7538	0.1811	4.164	**3.13E-05**
M. amazonicum	**Interaction**	2.7512	3.3652	0.818	0.413609					
nitrite x cypermethrin	**(Intercept)**	-1.5913	0.4958	-3.209	0.001331	**(Intercept)**	-1.8758	0.3664	-5.12	3.05E-07
	Barrage	48.5245	14.5267	3.34	**0.000837**	**Barrage**	59.2083	7.1043	8.334	**< 2E-16**
	Nitrite	0.8069	1.868	0.432	0.665764	**Nitrite**	2.0265	1.1261	1.8	0.0719
M. pantanalense	**Interaction**	48.227	59.0949	0.816	0.414447					
nitrite x cypermethrin	**(Intercept)**	-1.514	0.388	-3.903	9.50E-05	**(Intercept)**	-1.6658	0.2949	-5.649	1.61E-08
	Barrage	13.049	3.757	3.47E+00	**0.000515**	**Barrage**	15.0262	1.8111	8.297	**< 2E-16**
	Nitrite	-2.248	1.59	-1.414	0.157459	**nitrite**	-1.5601	1.0567	-1.476	0.14
M. amazonicum	**Interaction**	8.626	14.719	0.586	0.557859					

Table S2: Results of the linear regression analysis (pH or nitrite x cypermethrin-based Barrage®) of shrimp growth data (carapace length). Depending on the statistical significance of the interaction between both factors, the statistical analysis that best applies to each test is highlighted in grey. Statistically significant values are highlighted in bold.

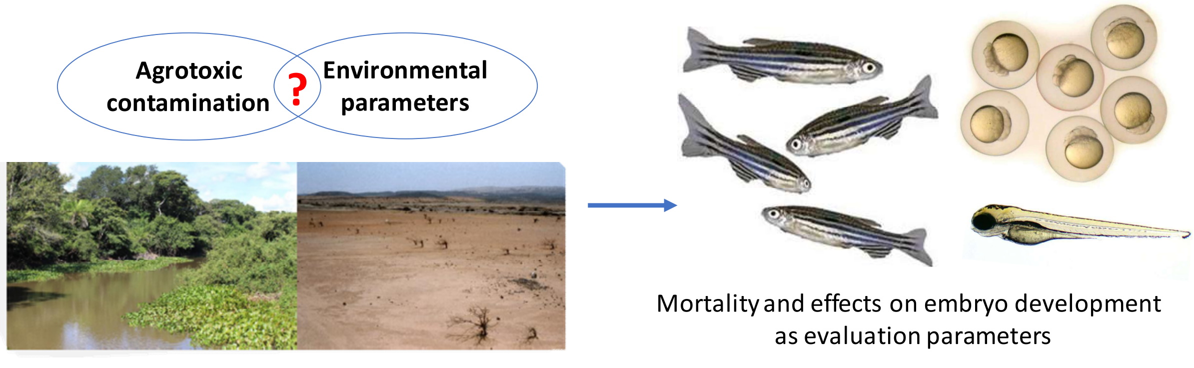
	Model with interaction	Estimate	Std. Error	Z value	P value	No interaction model	Estimate	Std. Error	Z value	P value
pH x cypermethrin	**(Intercept)**	1.27924	0.09785	13.074	< 2E-16	**(Intercept)**	1.05342	0.08291	12.706	< 2E-16
	Barrage	-0.0505	0.01392	-3.628	**0.000361**	**Barrage**	-0.01912	0.01193	-1.603	0.11052
M. pantanalense	**pH**	-6.02348	1.38762	-4.341	**0.0000223**	**pH**	-0.49119	0.15323	-3.206	**0.00156**
	Interaction	0.72005	0.17958	4.01	**0.0000852**					
pH x cypermethrin	**(Intercept)**	0.93477	0.043577	21.451	<2E-16	**(Intercept)**	**0.955362**	**0.032096**	**29.765**	**< 2E-16**
	Barrage	-0.003672	0.00584	-0.629	0.53	**Barrage**	**-0.006469**	**0.004252**	**-1.521**	**0.129**
M. amazonicum	**pH**	0.084114	1.013432	0.083	0.934	**pH**	**-0.622436**	**0.082031**	**-7.588**	**6.85E-13**
	Interaction	-0.097235	0.139009	-0.699	0.485					
nitrite x cypermethrin	**(Intercept)**	0.99088	0.01114	88.926	< 2E-16	**(Intercept)**	0.98497	0.00944	104.338	< 2E-16
	Barrage	-0.14073	0.0426	-3.304	**0.001245**	**Barrage**	-0.11607	0.03473	-3.342	**0.00109**
	Nitrite	-1.67723	0.43988	-3.813	**0.000215**	**Nitrite**	-1.28922	0.207	-6.228	**6.47E-09**
M. pantanalense	**Interaction**	1.6931	1.69364	1	0.319394					
nitrite x cypermethrin	**(Intercept)**	0.858191	0.009766	87.879	< 2E-16	**(Intercept)**	0.86401	0.007926	109.015	< 2E-16
	Barrage	0.325161	0.036499	8.909	**< 2E-16**	**Barrage**	0.300763	0.027566	10.91	**< 2E-16**
	nitrite	-0.460435	0.149484	-3.08	**0.00228**	**nitrite**	-0.594417	0.071316	-8.335	**3.84E-15**
M. amazonicum	**Interaction**	-0.555014	0.544229	-1.02	0.30872					

Table S3: Results of the logistic regression analysis (pH or nitrite x cypermethrin-based Barrage®) of shrimp developmental data. Depending on the statistical significance of the interaction between both factors, the statistical analysis that best applies to each test is highlighted in grey. Statistically significant values are highlighted in bold.

	Model with interaction	Value	Std. Error	t value	P value	No interaction model	Value	Std. Error	t value	P value
***pH x* cypermethrin**	**Barrage**	-153.234	0.207279	-739.2638	**0**	**Barrage**	-221.56926	32.84256	-6.746406	**1.516E-11**
	pH	2.33152	0.635947	3.666215	**0.0002462**	**pH**	0.8537243	0.356324	2.395919	**0.0165788**
M. pantanalense	**Interaction**	-21.92517	5.886033	-3.724949	**0.0001954**					
***pH x* cypermethrin**	**Barrage**	-33.17149	54.05867	-0.61362	0.5394663	**Barrage**	-38.029158	5.908986	-6.435818	**1.228E-10**
	pH	-0.906931	0.274031	-3.309595	**0.0009343**	**pH**	-0.9229131	0.17845	-5.171825	**2.318E-07**
M. amazonicum	**Interaction**	-0.607425	7.290043	-0.083323	0.9335951					
***Nitrite x* cypermethrin**	**Barrage**	-140.7855	26.34077	-5.344775	9.053E-08	**Barrage**	-103.4902	16.30036	-6.3489502	**2.168E-10**
	Nitrite	-3.51434	1.916724	-1.833514	0.0667262	**nitrite**	-1.526407	1.586296	-0.9622462	0.3359259
M. pantanalense	**Interaction**	153.588	79.61976	1.929019	0.0537286					
***nitrite x* cypermethrin**	**Barrage**	-87.59609	14.14588	-6.192337	5.928E-10	**Barrage**	-30.084705	3.663948	-8.2110086	**2.193E-16**
	nitrite	0.9748059	1.983675	0.4914141	0.6231336	**nitrite**	7.9230856	1.348433	5.8757734	**4.209E-09**
M. amazonicum	**Interaction**	173.39143	37.95588	4.568236	**4.918E-06**					

Chapter 3

Influence of pH and nitrite on the effects of cypermethrin on Zebrafish embryos



Effects of pH and nitrites on the toxicity of a cypermethrin-based pesticide in zebrafish embryos

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Summary

Physical and chemical water parameters can influence the toxicity of environmental contaminants by affecting their degradation, transformation and bioavailability. Therefore, it is important to understand how environmental factors, such as pH or nitrite concentrations, interact with chemical stressors (such as pesticides) that may reach the aquatic environment to allow a correct environmental impact assessment. The objective of this study was to evaluate the effects of pH and nitrite variation on the toxicity of the cypermethrin based pesticide Barrage®. Zebrafish embryos (*Danio rerio*) were exposed to a combination of pH or nitrite and cypermethrin through the Barrage® formulation for 96 hours and lethal and developmental endpoints assessed. At lethal level pH seemed to increase Barrage® toxicity, which was not expected given that hydrolysis rate of cypermethrin (the active compound of Barrage®) is higher at higher pHs. It is hypothesized that degradation products or other constituents of the formulation with increased toxicity may explain this result. Nitrite concentrations tested did not seem to change Barrage® toxicity. At sublethal level either for combinations involving pH or nitrites results were not very clear and generally lack statistical significance. Further work should be performed increasing the sample number in the experimental design. However, some trends suggest interactive effects of environmental and chemical factors at developmental level highlighting the need of further research. Sublethal endpoints must then be included in the assessment of combined effects of compounds given that they can furnish important information not obtained through lethality assessment.

Keywords: Pesticides; Barrage®; *Danio rerio*, Environmental Risk.

1. Introduction

In the last years, temperature rise has been the most emphasized consequence of the global climate changes (Harstad, 2016). This phenomenon however, is not dissociated from other effects including modification of hydrological regimes and precipitation patterns which influence water physicochemical parameters (Andrade et al., 2017). Parameters such as pH and nitrite concentrations may thus vary under climate change scenarios mainly due to the increase of the frequency of extreme events such as floods and prolonged droughts which modify the dilution capacity of rivers, lakes or streams (Marengo, 2008; Pereira et al., 2010; Ramsar, 2010). Significant variations in these parameters may become an additional risk factor for aquatic organisms, not only because of exceeding their tolerance limits but also due to the possible interactions with contaminants. For instance, several studies account for the influence of pH variation in the toxicity of aluminum (Dietrich and Schlatter, 1989); of the herbicide Vision (Chen et al., 2004), of the Glyphosate-based herbicide Roundup® (Tsui and Chu, 2003) and of fluoxetine (Nakamura et al., 2008). Many of these effects can be related to changes in the degradation rate, bioaccumulation or bioavailability of the compound (Dietrich and Schlatter, 1989; Maund et al., 2002). These studies indicate that variations of pH can change the predicted toxicity of chemical compounds, compromising environmental risk evaluations and preservation of ecosystems (Dietrich and Schlatter, 1989; Maund et al., 2002). Concerning nitrites, their influence in the toxicity of contaminants is scarcely known. Nitrites may interfere with the organisms' vital physiological and metabolic processes; a high nitrite load can cause changes on fish ionic regulatory, respiratory, cardiovascular, endocrine and excretory processes, as well as on the oxidation of hemoglobin in methemoglobin (Kroupova et al., 2005). The increase of nitrite levels in aquatic environments coming from industrial and urban sewage, aquaculture production or agriculture is of concern because of their potential toxicity (Jensen, 2003) and their interaction with other contaminants.

Pesticides are of special concern given their widespread use and their likely possibility of reaching the aquatic environments by direct application or by leaching and run off from the application sites. Once in the aquatic systems pesticides can accumulate in the environment and affect non-target organisms such as crustaceans and fish (Alpizar et al., 2011; Gowland et al., 2002; Ross and Sanches, 2006; Tu et al., 2010). Cypermethrin is a pyrethroid insecticide that acts as a neurotoxin that rapidly affects the central nervous system of insects, being highly toxic to invertebrates (e.g, aquatic insects and bees) and vertebrates (fish) (Keith and Walker, 1992; NPTN, 1998). Cypermethrin is the active ingredient of the pesticide formulation Barrage®, which is widely used in Brazil in agriculture, livestock production and domestic proposes (Barros, 1992; Gomes et al., 2011; NPTN, 1998). In addition, cypermethrin is used in aquaculture for parasites (such as *Lepeophtheirus salmonis*) control (Geferson et al., 2015; Hart et al., 1997; Luvizotto-Santos et al., 2009; Martins, 2004; Montanha and Pimpão, 2012; Tu et al., 2010).

The aim of this study was to evaluate the influence of pH and nitrite concentrations on Barrage® toxicity using zebrafish (*Danio rerio*) as model organism and using lethal and developmental endpoints. Zebrafish has been widely used in various toxicological assays due to its easy cultivation, reproduction and high egg production in laboratory conditions (Beliaeva et al., 2010; Zhang et al., 2015). The transparency of zebrafish eggs allows the easy observation of important developmental endpoints, including edema, tail deformation, developmental delay, hatching, among other parameters (Lammer et al., 2009; Williams et al., 2016). Moreover, the fish embryo toxicity test (FET) is an alternative for acute fish testing since embryonic stages until free feeding are not protected by the European directive for animal welfare (Braunbeck et al., 2005).

2. Material and methods

2.1 Zebrafish culture and egg production

D. rerio embryos were supplied by the Zebrafish facility at the Biology Department of the University of Aveiro, Portugal. Adult organisms were kept under controlled conditions, in a ZebTEC recirculation system (Tecniplast). The culture water was tap water filtered with activated charcoal and reverse osmosis, complemented with "Instant Ocean Synthetic Sea Salt" (Spectrum Brands, USA) and automatically adjusted pH and conductivity values. The water temperature was 27.0 ± 1 °C, conductivity 794 ± 50 µS/cm, pH of 7.5 ± 0.5 and dissolved oxygen equal to or greater than 95% saturation. The photoperiod cycle was maintained as 14h: 10h (light: dark). Adult fish were fed once daily with commercially available artificial diet Gemma Micro 500 (Skretting®, Spain). Wild type AB fish were used in all trials.

Zebrafish eggs were collected within 30 min after natural mating and rinsed in fish system water. Males and females were placed for crossing in aquariums (natural mating) as described in Andrade et al., (2016). Eggs were collected immediately after fertilization. Eggs were screened using a stereomicroscope (Stereoscopic Zoom Microscope-SMZ 1500, Nikon Corporation) and unfertilized or injured eggs excluded.

2.2 Acute toxicity to zebrafish embryos

Zebrafish eggs were exposed to combinations of an environmental parameter (pH or nitrite) and Barrage® following a full factorial design with 6 cypermethrin concentrations (0.0, 0.5, 1.5, 4.5, 13.5, 40.5 mg/L) and with 3 pH levels (6.0, 7.5 and 9.0) or 3 nitrite concentrations (57, 98 and 167 mg/L). The tests were performed on 6-well polyethylene microplates with 5 replicates per treatment. Each replicate consisted of 5 embryos in 10 ml solution. Embryos were exposed for 96 hours and daily observed in a stereoscopic microscope (Zoom-SMZ 1500, Nikon Corporation) for mortality recording. Test solutions were renewed daily.

2.3 Developmental toxicity to zebrafish embryos

In this test, lower concentrations of cypermethrin were used to evaluate sub lethal effects on embryonic development. The tests were performed on 24-well polyethylene microplates based on the OECD testing guideline 236 (OECD, 2013). Each treatment consisted of 20 embryos per concentration, 1 embryo in 2 ml of solution. Combinations of an environmental parameter (pH or nitrite) and Barrage® following a full factorial design with a 3 x 6: 6 cypermethrin concentrations (0.00, 0.01, 0.02, 0.06, 0.17, 0.50 mg/L) combined with 3 pH levels (6.0, 7.5 and 9.0) or 3 nitrite concentrations (57, 98 and 167 mg/L). The test lasted 96 hours. During this period, the test solutions was renewed daily. Fish embryos were observed daily using a stereoscopic microscope, and the following parameters were observed: hatching, tail bending, heart edema, lateral position (embryos unable to keep an upright position), yolk edema and delay in sac absorption (yolk sac noticeably larger in relation to the control).

2.4 Preparation of test solutions

Solutions of Barrage® at different pH

Zebrafish culture water with different pH was prepared using specific buffers (from Sigma-Aldrich®) for pH stabilization. For pH 6.0 the 2- (N-Morpholino) ethanesulfonic acid hydrate, 4-Morpholineethanesulfonic acid (MES) buffer was used; for pH 7.5 the 3- (N-Morpholino) propanesulfonic acid, 4-Morpholinepropanesulfonic acid (MOPS) buffer was used; and for pH 9.0 the 2- (Cyclohexylamino) ethanesulfonic acid (CHES) buffer was used. Buffers were diluted in fish culture water and pH adjusted with hydrochloric acid - HCl or sodium hydroxide - NaOH and using a portable multi-parameter device (Consort C5020). In order to attain the desired concentrations of cypermethrin, a small amount of the Barrage® stock solution was added to the pH-corrected water. Barrage® (Zoetis-Fort Dodge, Campinas, SP, Brazil) is a concentrated, emulsifiable suspension containing 150 g/L of cypermethrin (α-cyano-3-phenoxybenzyl-2,2-dimethyl-3- (2,2-dichlorovinyl) -cyclopropanecarboxylate, $C_{22}H_{19}Cl_2NO_3$, CAS number: 52315-07-8). Stock solution was prepared by diluting the formulation in zebrafish culture water.

Solutions of Barrage® with different nitrite concentrations

A stock solution of 1000 mg/L of nitrite was prepared from the dilution of sodium nitrite ($NaNO_2$) in distilled water. This solution was used to prepare the nitrite concentrations 57, 98 and 167 mg/L of the salt, diluted in fish culture water. Water with corrected nitrite concentrations was used to dilute the Barrage® stock solution and attain the desired cypermethrin concentrations.

To confirm nitrite concentrations of the prepared solutions, the nitrite content was determined using the NitriVer 3 Diazotization Method from Hach Co. (Loveland, CO) 8507. The HACH DR 2000 spectrometer was zeroed with deionized water. Twenty 5 ml of the sample were added to the Nitriver 3 reagent and stirred. The formation of a pink complex indicated the presence of nitrite. The reaction time was 15 minutes. Blank and samples were read at 585 nm.

2.5 Statistical analyses

The calculation of the LC_{50} values was performed by probit analysis in the statistical software package Minitab 17 (Minitab 17 Statistical Software, 2010).

Logistic regression models were built for all the binary variables (mortality, hatching, tail bending, heart edema, lateral position, yolk edema and delay in sac absorption using cypermethrin concentration and pH / nitrite concentration as fixed factors. To address the possible influence of environmental factors in cypermethrin toxicity the interaction term (Cypermethrin x pH or Cypermethrin x Nitrite) was also included in every model. The analyses were performed using R 3.5.1 software (R Core Team, 2018).

3. Results

3.1 Combination of pH and Barrage®

The combined effects of Barrage® and pH on mortality of zebrafish (*D. rerio*) larvae are shown in Table 1 (LC_{50} values), Fig 1 A and Fig S1 (supplementary material). An increased lethal toxicity was observed with increasing pH in zebrafish larvae (96 h-LC_{50} of 3.34 mg/L at pH 6.0; and 0.43 mg/L at pH 9.0). Statistical analysis however did not show a significant interaction (p= 0.207) between the 2 factors probably due to the high standard error associated (Table 2).

Table 1 - LC_{50} values, standard error (SE) and 95 % confidence interval for cypermethrin-based Barrage® toxicity to zebrafish at three different pH levels. Four days of exposure.

pH	LC_{50} (mg/L) / SE	Confidence interval 95%	
		Inferior limit	Upper limit
6.0	3.343 ± 0.696	2.147	5.369
7.5	1.340 ± 0.529	0.042	2.567
9.0	0.433 ± 0.475	-1.076	1.255

The combined effects of cypermethrin and pH levels on the embryonic development of zebrafish are depicted in Fig 1and S1 and Table 2. pH variation seemed to change the response to the chemical compound in several of the parameters tested. For instance hatching (Fig 1B) showed a very irregular pattern or response depending on pH; heart edema (Fig 1D) and delay in yolk sac absorption (Fig 1G) showed to increase at pH 6; tail bending (Fig 1C) seems to occur predominantly at pH values more distance from neutrality (pH 6 and 9) while higher frequency of yolk sac edemas (Fig 1F) were observed at pH 7.5. Statistical analysis however, only proved the existence of interaction for hatching and lateral position (Table 2). For most of the other parameters standard error was similar or even higher than the estimate value, preventing the robustness of the analysis.

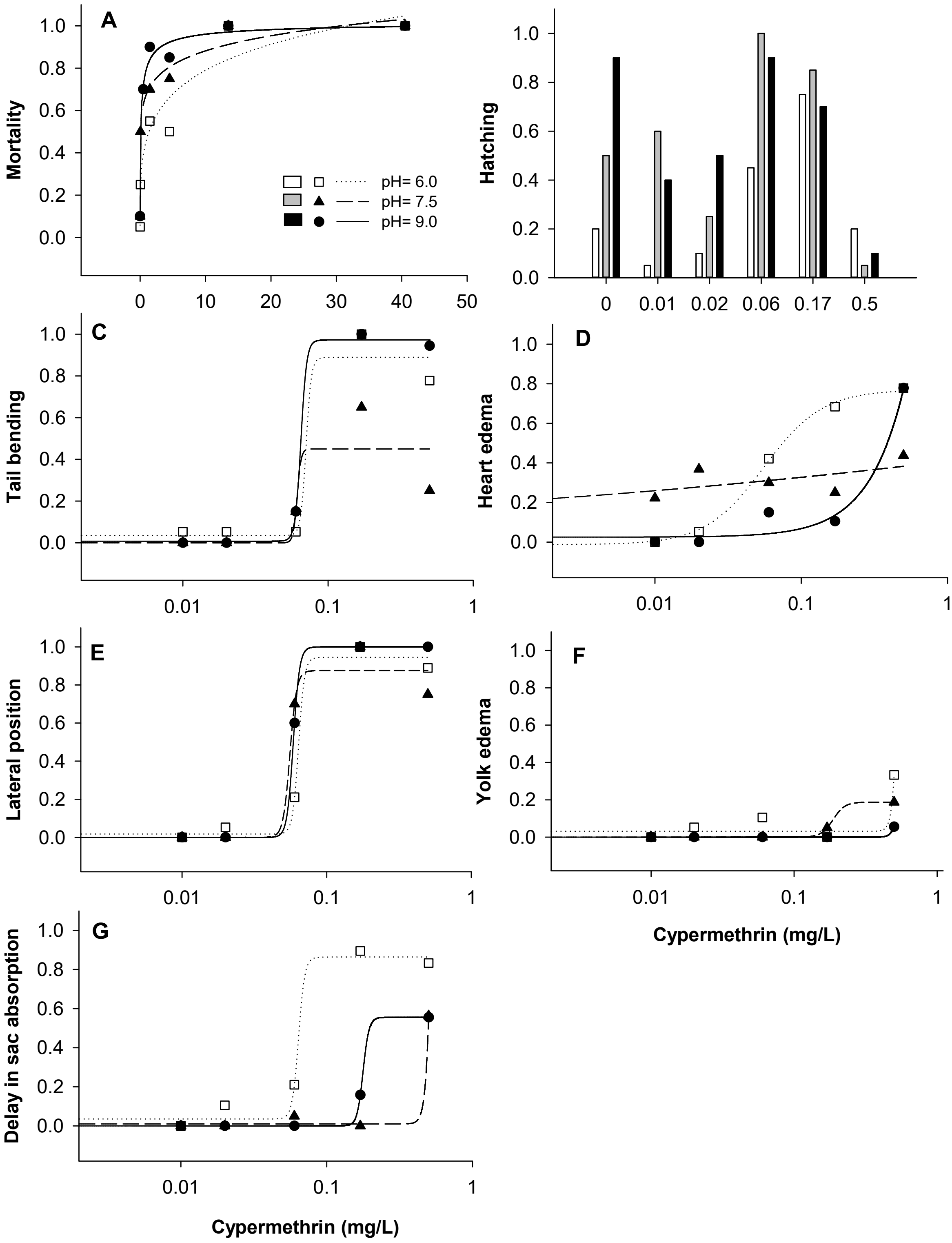

Figure 1. Effects of Barrage® at three pH levels on lethal and sublethal parameters: mortality (A); hatching (B), tail bending (C); heart edema (D); lateral position (E); yolk edema (F); delay in sac absorption (G). The values are means. Curve fit model = log-logistic four parameters function.

Table 2 - Effects of cypermethrin-CYP (via the Barrage® formulation) in combination with different pH levels in the embryonic development of zebrafish. Statistically significant differences are highlighted in bold.

		Estimate	Std. Error	z value	Pr(>z)
Mortality	(Intercept)	-3.325	1.124	-2.958	0.003
	CYP	-0.143	0.484	-0.296	0.767
	pH	0.343	0.148	2.316	**0.021**
	CYP x pH	0.089	0.070	1.261	0.207
Hatching	(Intercept)	-4.721	0.886	-5.327	0.000
	CYP	12.382	4.269	2.901	**0.004**
	pH	0.660	0.118	5.607	**0.000**
	CYP x pH	-2.025	0.578	-3.501	**0.000**
Tail bending	(Intercept)	-1.788	1.176	-1.520	0.129
	CYP	0.991	5.687	0.174	0.862
	pH	-0.033	0.156	-0.215	0.830
	CYP x pH	0.932	0.784	1.189	0.235
Heart edema	(Intercept)	1.071	1.181	0.907	0.365
	CYP	3.348	4.784	0.700	0.484
	pH	-0.422	0.164	-2.574	**0.010**
	CYP x pH	0.369	0.632	0.584	0.559
Lateral position	(Intercept)	-1.363	1.316	-1.035	0.301
	CYP	-17.947	14.384	-1.248	0.212
	pH	-0.105	0.176	-0.595	0.552
	CYP x pH	5.161	2.063	2.501	**0.012**
Yolk edema	(Intercept)	4.010	5.550	0.723	0.470
	CYP	-1.806	12.297	-0.147	0.883
	pH	-1.288	0.877	-1.468	0.142
	CYP x pH	1.313	1.911	0.687	0.492
Delay in sac absorption	(Intercept)	5.259	2.222	2.366	0.018
	CYP	3.484	6.764	0.515	0.607
	pH	-1.205	0.343	-3.514	**0.000**
	CYP x pH	0.849	0.938	0.906	0.365

3.2 Combination Nitrite x Barrage®

A logistic regression to assess the effects of the two factors individually was first done (Table 3) given that no information about the effects of nitrites on zebrafish embryo development was available. Results showed that nitrite concentrations had a significant effect on the hatching, tail bending and heart edema. A logistic regression accounting for the interaction of Barrage® and nitrites was then performed (Table 4; see also Fig S2 for effects estimated by the logistic regression model). Nitrites did not change lethal toxicity of Barrage® (Fig 2A). LC_{50} values (Table 5) were similar among the nitrite concentrations tested and no interaction was verified in the statistical analysis (Table 4). Hatching (Fig 2B) seemed to increase with concentrations on nitrites however in a not very clear pattern; tail bending (Fig 2C) and heart edema (Fig 2D) seemed to be increased in the lowest concentration of nitrite tested and lateral position effect (Fig 2E) seemed to be more relevant at intermediary nitrite concentrations. However, statistical analysis only revealed a significant interaction for the endpoints lateral position. For most of the other parameters the standard error value is too high compared to the estimate value (Table 4) preventing the discrimination of significant effects.

Table 3- Analysis of the effects of Barrage® and nitrites concentrations in the mortality and embryo development of zebrafish using a logistic regression model without testing for interaction. Bold values highlight significant effects.

		Estimate	Std. Error	z value	Pr(>|z|)
Mortality	(Intercept)	-3.238	0.499	-6.482	0.000
	CYP	0.427	0.048	8.851	**0.000**
	NIT	0.003	0.002	1.182	0.237
Hatching	(Intercept)	-1.382	0.274	-5.043	0.000
	CYP	0.446	0.593	0.752	0.452
	NIT	0.006	0.001	4.417	**0.000**
Tail bending	(Intercept)	-1.374	0.392	-3.499	0.000
	CYP	11.698	1.373	8.520	**0.000**
	NIT	-0.006	0.002	-2.716	**0.006**
Heart edema	(Intercept)	-1.489	0.473	-3.148	0.001
	CYP	4.790	0.836	5.726	**0.000**
	NIT	-0.010	0.003	-3.316	**0.001**
Lateral position	(Intercept	-2.577	0.539	-4.775	0.000
	CYP	48.422	6.554	7.387	**0.000**
	NIT	-0.005	0.003	-1.651	**0.098**
Delay in sac absorption	(Intercept)	-1.636	0.363	-4.502	0.000
	CYP	5.415	0.692	7.815	**0.000**
	NIT	-0.003	0.002	-1.608	0.108

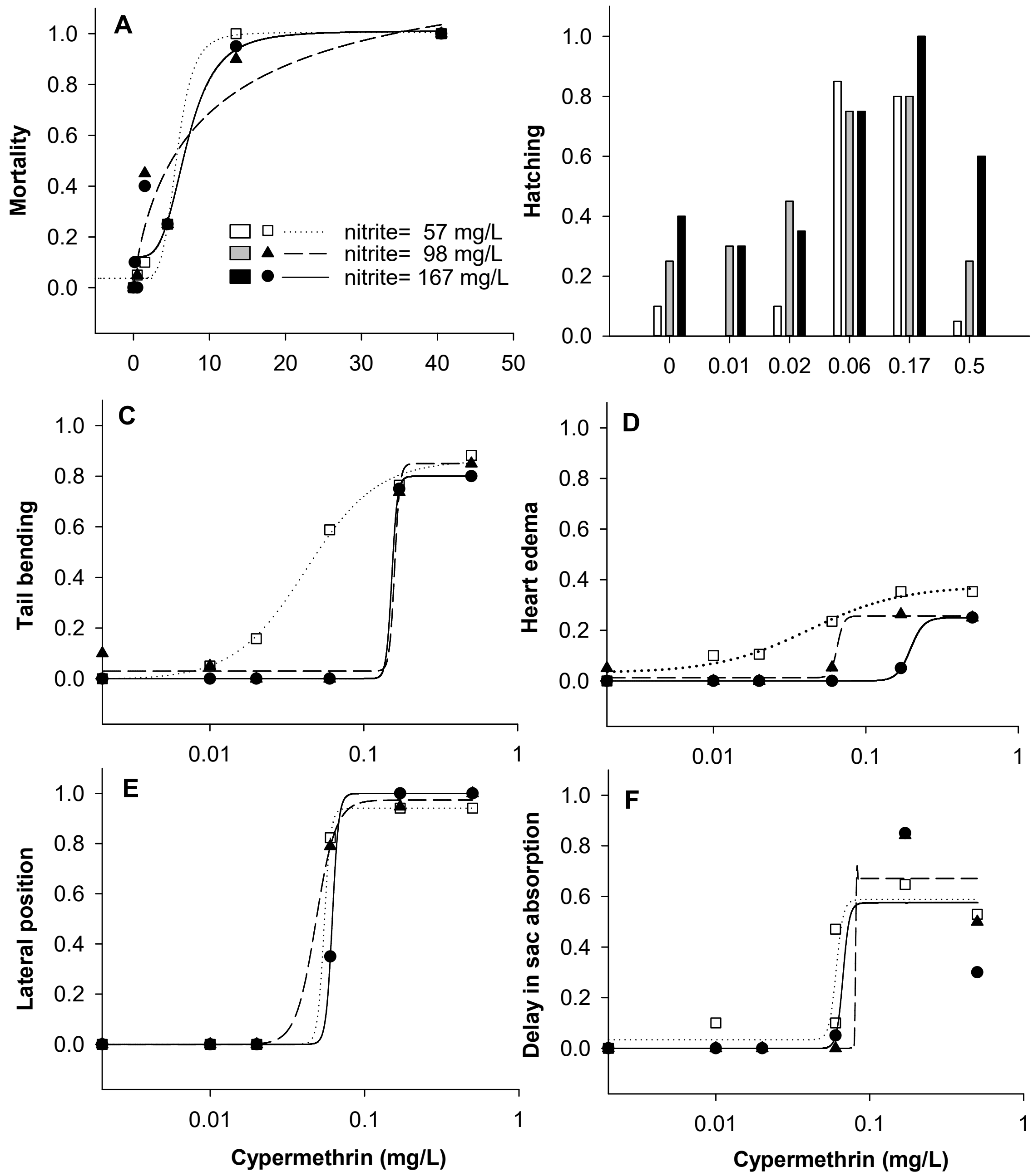

Figure 2. Effects Barrage® at three concentration of nitrite on lethal and sublethal endpoints: mortality (A); hatching (B); tail bending (C); heart edema (D); lateral position (E); delay in sac absorption (F). The values are means. Curve fit model = log-logistic four parameters function.

Table 4- Analysis of the effects of Barrage® and nitrites concentrations in the mortality and embryo development of zebrafish using a logistic regression model testing for interaction. Bold values highlight significant effects.

		Estimate	Std. Error	z value	Pr(>z)
Mortality	(Intercept)	-3.682	0.651	-5.654	0.000
	CYP	0.566	0.138	4.092	**0.000**
	NIT	0.006	0.003	1.643	0.100
	CYP x NIT	-0.001	0.001	-1.150	0.250
Hatching	(Intercept)	-1.117	0.313	-3.564	0.000
	CYP	-2.087	1.648	-1.267	0.205
	NIT	0.005	0.002	2.819	**0.005**
	CYP x NIT	0.016	0.009	1.653	0.098
Tail bending	(Intercept)	-1.400	0.495	-2.825	0.005
	CYP	11.983	3.744	3.201	**0.001**
	NIT	-0.007	0.003	-2.097	**0.036**
	CYP x NIT	-0.002	0.020	-0.082	0.935
Heart edema	(Intercept)	-0.421	0.744	-0.565	0.572
	CYP	0.771	2.174	0.355	0.723
	NIT	-0.020	0.006	-3.091	**0.002**
	CYP x NIT	0.030	0.016	1.927	0.054
Lateral position	(Intercept)	0.621	1.290	0.481	0.630
	CYP	-19.556	21.796	-0.897	0.370
	NIT	-0.034	0.013	-2.607	**0.009**
	CYP x NIT	0.582	0.221	2.636	**0.008**
Delay in sac absorption	(Intercept)	-1.754	0.459	-3.825	0.000
	CYP	6.131	1.834	3.344	**0.001**
	NIT	-0.003	0.003	-0.955	0.340
	CYP x NIT	-0.004	0.010	-0.424	0.672

Table 5 - LC_{50} values, standard error (SE) and 95 % confidence interval for cypermethrin-based Barrage® toxicity to zebrafish at three different nitrite concentrations. Four days of exposure.

Nitrito (mg/L)	LC_{50} (mg/L) / SE	Confidence interval 95%	
		Inferior limit	Upper limit
57	6.023 ± 0.911	4.651	8.893
98	6.881 ± 0.910	5.351	9.201
167	6.302 ± 0.861	4.871	8.538

4. Discussion

This work aimed to evaluate the influence of environmental conditions, more specifically pH and nitrite, on the toxicity of Barrage® in zebrafish embryos. The individual toxicity of Barrage® was previously characterized by Soares et al. (2017). Similarly, effects of pH on the tested endpoints were reported by Andrade et al (2017) and based on this work no lethal or developmental effects are expected at the pH levels tested (6, 7.5 and 9). The 96h-LC_{50} value found in this study for zebrafish embryos at pH 7.5 (1.34 mg/L) is in the same order of magnitude of previously reported values (144h-LC50 of 1.60 mg/L) for the same cypermethrin base formulation (Soares et al., 2017). The results also suggest that the acute toxicity of cypermethrin increases at pH 9.0 for zebrafish larvae (although the variability of the data excludes the statistical confirmation of interaction). The toxicity of the pesticide may be influenced by pH variations due to changes in hydrolysis rates, promoting rapid or slow degradation of the compound, thereby altering the availability to the organisms (Chen et al., 2004). Cypermethrin at pH 8.38 shows a higher rate of hydrolysis, with a 31.2% degradation, compared with pH 6.0 where a degradation of 8% is observed (Al-Mughrabi et al., 1992; Extoxnet, 1996; Kamrin, 1997). In addition, the efficacy of cypermethrin in the control of the *Rhipicephalus microplus* tick is higher at reduced pH (Garcia, 2007). In the present study, Barrage® toxicity was however higher at pH 9 then at pH 6 or 7.5. This increased toxicity may be related to the degradation metabolites of the compound, the inert agents of the formulation and / or their interaction with different pHs. One of the most important metabolite of cypermethrin is 3-phenoxybenzoic acid, a compound also generated from the degradation of the pyrethroid permethrin, which may be more toxic to algae and bacteria in relation to the original compound; in addition, a synergistic effect between metabolites and permethrin has been observed (Stratton and Corke, 1982; Xie et al., 2008). Inert agents present in the Barrage® formulation, for example C9 aromatic compounds and a minority of C8 and C10 aromatics, may contribute to a higher toxicity of this formulation (Soares et al., 2017). Therefore, to better understand the increased toxicity of Barrage® at pH 9, tests with the individual components of the formulation should be performed.

In general, pH appeared to have changed the response to Barrage® in several sublethal endpoints, but data variability prevented the confirmation of the verified trends and thus conclusions about the effects of pH on Barrage® toxicity are possible only to a limited extent. Data variability can be decreased by increasing the sample number and thus, it is highly recommended to increase the N in future experiments of combined toxicity. Trends observed suggest that pH more distant from neutrality (6 and 9) increase the toxicity of cypermethrin (e.g. tail bending, yolk sac absorption and lateral position) but for other endpoints this is not clear. This is the case of hatching where effects were not dose-dependent. At concentrations of Barrage® and pH tested no effects on hatching rate were expected (Soares et al 2017, Andrade et al 2017). Thus, results point out to a possible interference of pH in Barrage® toxicity which should be further confirmed by an experimental design allowing better statistical robustness. Further studies related to the type and nature of the interaction between these two factors should be conducted, including understanding the response mechanisms of the compound at the biochemical level, to better explain the observed results. In the aquatic ecosystem pH directly interferes with the abundance and geographical distribution of organisms (Muniz, 2010) and its variation can reduce the reproductive efficiency of the species, as well as respiratory and locomotor functions, feeding ability, and alter cellular and molecular mechanisms (Fromm, 1980; Kwong et al., 2014; Oliveira and Goulart, 2000).

Nitrite is an important parameter of water quality that may interfere with multiple physiological functions. Jensen (2003) reviewed the effects of nitrites to aquatic organisms reporting the involvement of nitrites at several levels including ion regulation, respiration, cardiovascular function, endocrine regulation and excretion (Jensen, 2003). In this work, the nitrite concentrations tested did not cause zebrafish embryos mortality. Studies show that the acute toxicity of nitrite to zebrafish can vary according to age; a 96h-LC_{50} of 386.00 mg/L was calculated for 20-25 days old larvae while a 96h-LC_{50} of 242.41 mg/L was calculated for 2 to 3 months juveniles (Voslářová et al., 2006).

Individual effects of nitrites observed in this work (tail bending, heart edemas and effects on hatching) are probably linked to the above mentioned effects but further studies are needed to understand the mode of action at developmental level. The effects of nitrites in the initial development of the zebrafish were previously studied in a sublethal exposure for 96 hours. Authors observed absence of insufflation of the swimming bladder at 100 mg/L or higher, yolk sac edema, pericardial edema and craniofacial defects at 200 and 300 mg/L (Simmons et al., 2012). In addition, the authors observed immobilization of larvae and tail curvature at 200 and 300 mg/L, despite non-statistical verification due to variability of the data. In the literature, studies with zebrafish have also reported abnormal heart development, reduced body length and defective development of the nervous or muscular system associated with elevated levels of nitrite (100 mg/l sodium nitrite) (Li et al., 2014) and reduced growth after long-term zebrafish exposure to sublethal levels (73 mg/L) (Voslářová et al., 2008).

In our study, a significant effect of nitrite on cardiac edema was observed. In fact, studies at histological, cellular and molecular levels show that excess nitrite (100 mg/l sodium nitrite) causes malformation of the valvular structures, resulting in cardiac edema in zebrafish embryos (Li et al., 2014). The authors observed that excess nitrite disrupts the development of endocardial cells in the atrioventricular canal of embryos and decreases the expression of progenitor markers of the valve.

In this work, the nitrite levels tested do not appear to affect the acute toxicity (LC_{50}) of cypermethrin to zebrafish larvae. At the sublethal level, similarly to the observed for pH experiments, high variability of data prevented the discrimination of interaction in the statistical analysis in spite of some important trends observed. Thus, confirmation of results with an increased N is essential. General trends indicate, however, that effects are not dependent on nitrites concentrations with the lowest level tested (86 mg/L) resulting in some cases in higher Barrage® toxicity (this can be observed for instance in the tail bending).

Generally, results of this study suggest that both pH and nitrites may modify the toxicity of Barrage®, confirming the need of account for environmental variables in the assessment of chemicals effects. Further studies are, however, needed to confirm the trends observed. This study also suggested the importance of developmental parameters in the analysis of chemical effects given that the assessment of lethality may not be enough to characterize the combined effects of compounds. This is the case of Nitrite concentrations tested which did not appear to influence Barrage® responses at lethal level, but showed some effects at sublethal level. The effects at sublethal level, although not representing drastic effects that compromise the survival of the species (Zagatto and Bertoletti, 2014), may, in the long term, compromise its fitness and ecosystem equilibrium (Sih et al., 2004). This reinforces the need to take into account environmental conditions in the ecological risk assessment, taking into account the fluctuations of these parameters in water bodies, susceptible to more drastic variations of these conditions compared to the tests with optimal conditions. The use of different species and varied environmental parameters is recommended in the evaluation to avoid overestimation of the risk.

5. Conclusion

In this study combined effects of the pesticide Barrage® and two environmental parameters (pH and nitrites) were tested using Zebrafish embryos. At lethal level pH seemed to increase Barrage® toxicity, which was not expected given that hydrolysis rate of cypermethrin (the active compound of Barrage®) is higher at higher pHs. It is hypothesized that degradation products or other constituents of the formulation with increased toxicity may explain this result. Nitrite concentrations tested did not seem to change Barrage® toxicity. At sublethal level either for combinations involving pH or nitrites results were not very clear and generally lack statistical significance. Further work should be performed increasing the sample number in the experimental design. However, some trends suggest interactive effects of environmental and chemical factors at developmental level highlighting the need of further research. Sublethal endpoints must then be included in the assessment of combined effects of compounds given that they can furnish important information not obtained through lethality assessment.

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6. References

Al-Mughrabi, K.I., Nazer, I.K., Al-Shuraiqi, Y.T., 1992. Effect of pH of water from the King AbdaHah Canal in Jordan on the stability of cypermethrin. Crop Prot. 11, 341–344. https://doi.org/0261-219419210410341-04

Alpizar, F., Carlsson, F., Naranjo, M.A., 2011. The effect of ambiguous risk, and coordination on farmers' adaptation to climate change — A framed field experiment. Ecol. Econ. 70, 2317–2326. https://doi.org/10.1016/j.ecolecon.2011.07.004

Andrade, T.S., Henriques, J.F., Almeida, A.R., Machado, A.L., Koba, O., Giang, P.T., Soares, A.M.V.M., Domingues, I., 2016. Carbendazim exposure induces developmental, biochemical and behavioural disturbance in zebrafish embryos. Aquat. Toxicol. 170, 390–399. https://doi.org/10.1016/j.aquatox.2015.11.017

Andrade, T.S., Henriques, J.F., Almeida, A.R., Soares, A.M.V.M., Scholz, S., Domingues, I., 2017. Zebrafish embryo tolerance to environmental stress factors—Concentration–dose response analysis of oxygen limitation, pH, and UV-light irradiation. Environ. Toxicol. Chem. 36, 682–690. https://doi.org/10.1002/etc.3579

Barros, A. de, 1992. Recomendações para controle da mosca-dos-chifres no Pantanal. Embrapa, Centro de Pesquisa Agropecuária do Pantanal (Corumba, MS). [WWW Document]. ainfo.cnptia.embrapa.br.

Beliaeva, N.F., Kashirtseva, V.N., Medvedeva, N. V, Khudoklinova, I.I., Ipatova, O.M., Archakov, A.I., 2010. Zebrafish as a model organism for biomedical studies. Biomed. Khim. 56, 120–31.

Braunbeck, T., Böttcher, M., Hollert, H., Kosmehl, T., Lammer, E., Leist, E., Rudolf, M., Seitz, N., 2005. Towards an Alternative for the Acute Fish LC 50 Test in Chemical Assessment : The Fish Embryo Toxicity Test Goes Multi-species – an Update 87–102.

Chen, C.Y., Hathaway, K.M., Folt, C.L., 2004. Multiple stress effects of Vision® herbicide, ph, and food on zooplankton and larval amphibian species from forest wetlands. Environ. Toxicol. Chem. 23, 823. https://doi.org/10.1897/03-108

Dietrich, D., Schlatter, C., 1989. Aluminium toxicity to rainbow trout at low pH. Aquat. Toxicol. 15, 197–212. https://doi.org/10.1016/0166-445X(89)90036-2

Extoxnet, 1996. Pesticide Information Profiles - Cypermethrin [WWW Document]. URL http://extoxnet.orst.edu/pips/cypermet.htm (accessed 4.4.18).

Fromm, P. 0., 1980. A review of some physiological and toxicological responses of freshwater fish to acid stress. Env. BioL Fish 5, 79–93.

Garcia, F., Lúcio, N., 2007. Influência do ph do diluidor , na ação de caldas ixodicidas (Amitraz , Clorpirifós e Cipermetrina), contra *Rhipicephalus (Boophilus) microplus* (Canestrini , 1887) (Acarina : Ixodidae). Faculdade de Ciências Agrárias e Veterinárias – UNESP.

Geferson, A., Universidade, B., Helena, R., Mour, V., Federal, U., Biochemistry, A., Silva, L., 2015. Aquicultura no Brasil : Novas Perspectivas, ResearchGate. São Carlos, SP.

Gomes, A., Koller, W., Barros, A., 2011. Susceptibility of *Rhipicephalus* (Boophilus) *microplus* to acaricides in Mato Grosso do Sul, Brazil. Ciência Rural 41, 1447–1452. https://doi.org/http://dx.doi.org/10.1590/S0103-84782011005000105

Gowland, B.T.., Moffat, C.F., Stagg, R.M., Houlihan, D.F., Davies, I.M., 2002. Cypermethrin induces glutathione S-transferase activity in the shore crab, *Carcinus maenas*. Mar. Environ. Res. 54, 169–177. https://doi.org/10.1016/S0141-1136(02)00105-8

Harstad, B., 2016. The dynamics of Climate Agreements. J. Eur. Econ. Assoc. 14, 719–752. https://doi.org/10.1111/jeea.12138

Hart, J.L., Thacker, J.R., Braidwood, J.C., Fraser, N.R., Matthews, J.E., 1997. Novel cypermethrin formulation for the control of sea lice on salmon (*Salmo salar*). Vet. Rec. 140, 179–81. https://doi.org/10.1136/VR.140.7.179

Jensen, F.B., 2003. Nitrite disrupts multiple physiological functions in aquatic animals. Comp. Biochem. Physiol. Part A Mol. Integr. Physiol. 135, 9–24. https://doi.org/10.1016/S1095-6433(02)00323-9

Kamrin, M.A., 1997. Pesticide Profiles: Toxicity, Environmental Impact, and Fate, CRC press.

Keith, L.H., Walker, M., 1992. EPA'S Pesticide Fact Sheet Database. CRC Press.

Kroupova, H., Machova, J., Svobodova, Z., 2005. Nitrite influence on fish: a review. Vet. Med. 50, 461–471.

Kwong, R.W.M., Kumai, Y., Perry, S.F., 2014. The physiology of fish at low pH : the zebrafish as a model system. Co. Biol. 651–662. https://doi.org/10.1242/jeb.091603

Lammer, E., Carr, G.J., Wendler, K., Rawlings, J.M., Belanger, S.E., Braunbeck, T., 2009. Is the fish embryo toxicity test (FET) with the zebrafish (*Danio rerio*) a potential alternative for the fish acute toxicity test? Comp. Biochem. Physiol. Part C Toxicol. Pharmacol. 149, 196–209. https://doi.org/10.1016/J.CBPC.2008.11.006

Li, J., Jia, W., Zhao, Q., 2014. Excessive Nitrite Affects Zebrafish Valvulogenesis through Yielding Too Much NO Signaling. PLoS One 9, e92728. https://doi.org/10.1371/journal.pone.0092728

Luvizotto-Santos, R., Eler, M.N., Espindola, E.L.G., Vieira, E.M., 2009. O uso de praguicidas nas pisciculturas e pesqueiros situados na Bacia do rio Mogi-Guaçu. Bol. do Inst. Pesca 35, 343–358.

Marengo, J.A., 2008. Water and Climate Change. Estud. Avançados 22, 83–96. https://doi.org/http://dx.doi.org/10.1590/S0103-40142008000200006

Martins, M.L., 2004. Cuidados Básicos e Alternativas no Tratamento de Enfermidades de Peixes na Aqüicultura Brasileira, in: Editora Varela (Ed.), Sanidade de

Organismos Aquáticos. pp. 355–368.

Maund, S.J., Hamer, M.J., Lane, M.C.G., Farrelly, E., Rapley, J.H., Goggin, U.M., Gentle, W.E., 2002. Partitioning, bioavailability, and toxicity of the pyrethroid insecticide cypermethrin in sediments. Environ. Toxicol. Chem. 21, 9–15. https://doi.org/10.1002/etc.5620210102

Minitab 14 Statistical Software, 2010. https://doi.org/[Computer software]. State College, PA: Minitab, Inc. (www.minitab.com)

Montanha, F.P., Pimpão, C.T., 2012. Efeitos toxicológicos de piretróides (cipermetrina e deltametrina) em peixes-Revisão. Rev. Científica Eletrônica Med. Veterinária 18, 1–58.

Muniz, C.C., 2010. Avaliação do papel do pulso de inundação sobre a riqueza e biodiversidade de peixes em ambiente inundável, no sistema de baías caiçara, porção norte do Pantanal Matogrossense, alto Paraguai. Tese de doutorado. Universidade Federal de São Carlos.

Nakamura, Y., Yamamoto, H., Sekizawa, J., Kondo, T., Hirai, N., Tatarazako, N., 2008. The effects of pH on fluoxetine in *Japanese medaka* (*Oryzias latipes*): Acute toxicity in fish larvae and bioaccumulation in juvenile fish. Chemosphere 70, 865–873. https://doi.org/10.1016/j.chemosphere.2007.06.089

NPTN, 1998. National Pesticide Information Center -Cypermethrin. Oregon.

OECD, 2013. Test No. 236: Fish Embryo Acute Toxicity (FET) Test, OECD Guidelines for the Testing of Chemicals, Section 2. OECD Publ. 1–22. https://doi.org/doi:10.1787/9789264203709-en

Oliveira, E.F. de, Goulart, E., 2000. Distribuição espacial de peixes em ambientes lênticos : interação de fatores. Acta Sci. Biol. Sci. 22, 445–453. https://doi.org/10.4025/actascibiolsci.v22i0.2963

Pereira, G., Elisa, M., Silva, S., Moraes, E.C., 2010. Impactos climáticos das áreas alagadas no Bioma Pantanal. Embrapa Informática Agropecuária/INPE 190–199.

R Core Team, 2018. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. [WWW Document]. URL http//www.R-project.org/.

Ramsar, 2010. Cuidar das zonas úmidas - uma resposta às mudanças climáticas, Ramsar. Brasília - DF.

Ross, J., Sanches, L., 2006. Plano de conservação da bacia do alto Paraguai e o zoneamento ecológico-econômico para o Brasil. An. 1º Simpósio Geotecnologias no Pantanal, Campo Gd. Bras. 1, 11–15.

Sih, A., Bell, A.M., Kerby, J.L., 2004. Two stressors are far deadlier than one. Trends Ecol. Evol. 19, 274–276. https://doi.org/10.1016/J.TREE.2004.02.010

Simmons, A.E., Karimi, I., Talwar, M., Simmons, T.W., 2012. Effects of Nitrite on Development of Embryos and Early Larval Stages of the Zebrafish (*Danio rerio*). Zebrafish 9, 200–206. https://doi.org/10.1089/zeb.2012.0746

Soares, M.P., Jesus, F., Almeida, A.R., Zlabek, V., Grabic, R., Domingues, I., Hayd, L., 2017. Endemic shrimp *Macrobrachium pantanalense* as a test species to assess potential contamination by pesticides in Pantanal (Brazil). Chemosphere 168, 1082–1092. https://doi.org/10.1016/j.chemosphere.2016.10.100

Stratton, G.W., Corke, C.T., 1982. Toxicity of the insecticide permethrin and some degradation products towards algae and cyanobacteria. Environ. Pollut. Ser. A, Ecol. Biol. 29, 71–80. https://doi.org/10.1016/0143-1471(82)90055-1

Tsui, M.T.K., Chu, L.M., 2003. Aquatic toxicity of glyphosate-based formulations: comparison between different organisms and the effects of environmental factors. Chemosphere 52, 1189–1197. https://doi.org/10.1016/S0045-6535(03)00306-0

Tu, H.T., Silvestre, F., Phuong, N.T., Kestemont, P., 2010. Effects of pesticides and antibiotics on penaeid shrimp with special emphases on behavioral and biomarker responses. Environ. Toxicol. Chem. 29, 929–938. https://doi.org/10.1002/etc.99

Vosláŕová, E., Pištěková, V., Svobodová, Z., 2006. Nitrite Toxicity to *Danio rerio*: Effects of Fish Age and Chloride Concentrations. Acta Vet. Brno 75, 107–113. https://doi.org/10.2754/avb200675010107

Vosláŕová, E., Pištěková, V., Svobodová, Z., Bedáňová, I., 2008. Nitrite Toxicity to *Danio rerio*: Effects of Subchronic Exposure on Fish Growth. Acta Vet. Brno 77, 455–460. https://doi.org/10.2754/avb200877030455

Williams, M.B., Dennis, L., Miyasaki, N., Barry, R., Powell, M., Watts, S., Smith, D., 2016. Effect of Dietary Protein Source and Quantity on the Growth and Body Composition of Juvenile *Danio rerio*. FASEB J. 30, 915–28.

Xie, W.-J., Zhou, J.-M., Wang, H.-Y., Chen, X.-Q., 2008. Effect of Nitrogen on the Degradation of Cypermethrin and Its Metabolite 3-Phenoxybenzoic Acid in Soil. Pedosphere 18, 638–644. https://doi.org/10.1016/S1002-0160(08)60058-2

Zagatto, P.A., Bertoletti, E., 2014. Ecotoxicologia Aquática Princípios e Aplicações, 2nd ed. São Carlos, SP.

Zhang, Q., Cheng, J., Xin, Q., 2015. Effects of tetracycline on developmental toxicity and molecular responses in zebrafish (*Danio rerio*) embryos. Ecotoxicology 24, 707–719. https://doi.org/10.1007/s10646-015-1417-9

Supplementary Data

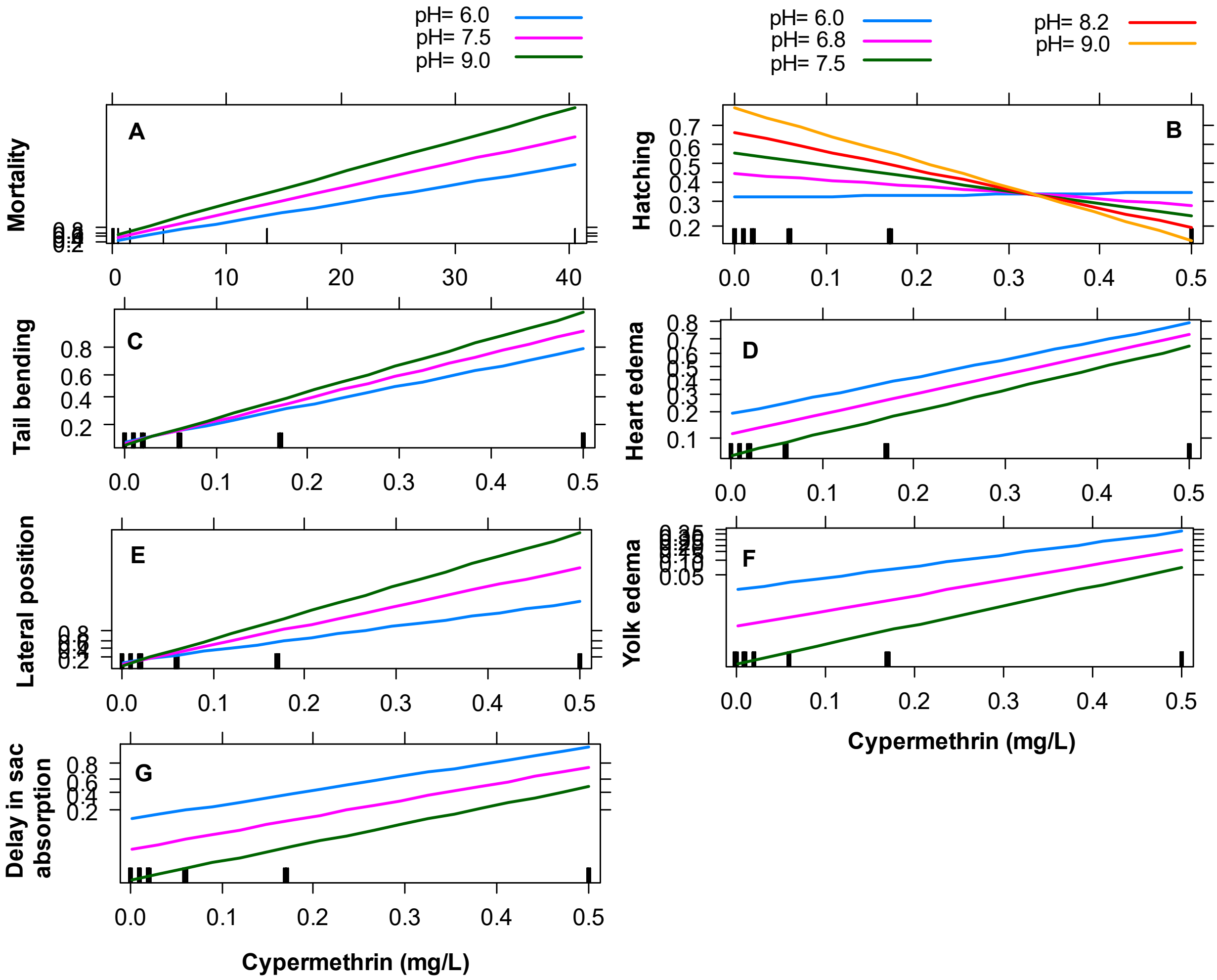


Figure S1. Effects estimated by the logistic regression model for cypermethrin (mg/L) in combination with pH concentrations in zebrafish larvae: mortality at day 4 (A); hatching (B); tail bending (C); heart edema (D); lateral position (E); yolk edema (F); delay in sac absorption (G).

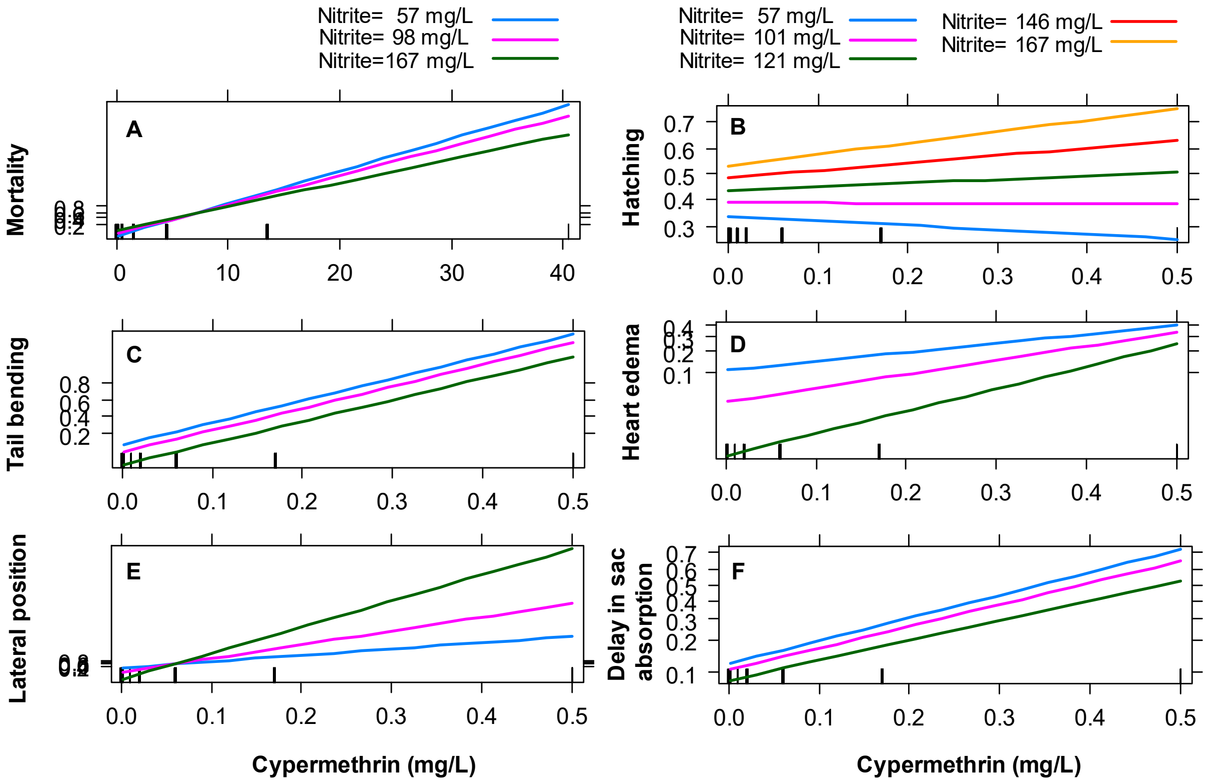
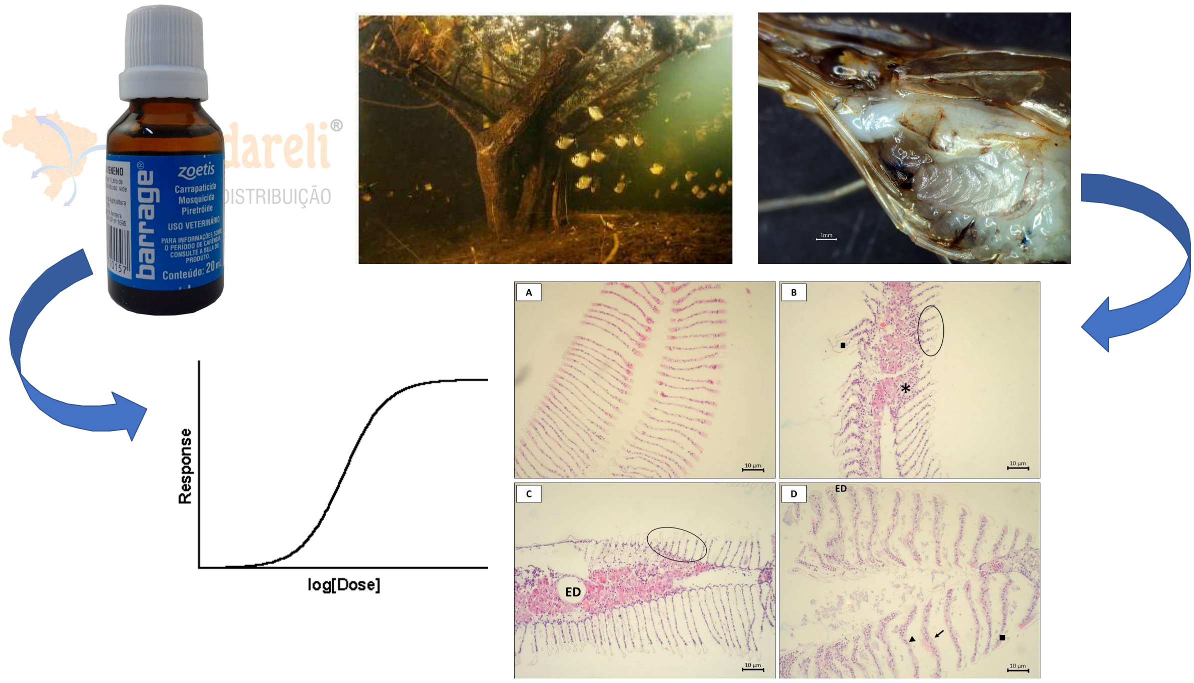


Figure 2. Effects estimated by the logistic regression model for cypermethrin (mg/L) in combination with nitrite concentrations in zebrafish larvae: mortality at day 4 (A); hatching (B); tail bending (C); heart edema (D); lateral position (E); delay in sac absorption (F).

Chapter 4

Cypermethrin-based formulation Barrage® induces histological changes in gills of the Pantanal endemic shrimp *Macrobrachium pantanalense*



Cypermethrin-based formulation Barrage® induces histological changes in gills of the Pantanal endemic shrimp *Macrobrachium pantanalense*

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Abstract

Pantanal shrimp *Macrobrachium pantanalense* was exposed for 96 hours to the cypermethrin-based formulation Barrage®. Population-relevant endpoints (survival, swimming behavior) as well as histopathology of gills were analyzed. A 96 h-LC_{50} of 0.93 µg/L of cypermethrin was calculated while equilibrium disturbances were observed at 1.25 µg/L. Histological examination showed predominantly regressive changes in the gills of shrimp exposed to concentrations of 0.25 and 1.25 µg/L. Three levels of lesions were observed in the gills: I- Intercellular edema, epithelial lifting of the lamellae and lamellar fusion, fat vacuoles and hypertrophy of gill epithelial cells or mucous cells; II- nuclear changes, atrophy (reduction of volume and number) and hyperplasia of gill epithelia and III- necrosis. This study shows the high sensitivity of the shrimp *M. pantanalense* to the pesticide Barrage® highlighting the importance of monitoring agrochemicals residues in the Pantanal region (Brazil) and conduct risk evaluation studies to prevent deleterious effects on the aquatic communities of Pantanal.

Keywords: pesticide; crustaceans; histopathology; behavior; wetlands; environmental risk.

1. Introduction

The Pantanal (Brazil) is a biosphere reserve consisting of seasonally flooded areas that hold a rich and admirable diversity and abundance of wild species, some of which are listed as threatened or endangered (Junk et al., 2006). Particularly, a significant diversity of freshwater shrimp of the genus *Macrobrachium* (Crustacea: Decapoda: Caridea: Palemonidae) (Murphy and Austin, 2005) occurs in Pantanal. This diversity is progressively growing due to the increase in the number of studies describing new species in the region (De Grave et al., 2008). This is the case of *Macrobrachium pantanalense*, a newly described freshwater shrimp endemic to Pantanal. Until recently, it was classified as *M. amazonicum*, a species widely distributed throughout South America (Melo, 2003; Santos et al., 2013).

Recent development in agriculture and livestock sectors in the region led to an increased use of pesticides, as a mean to ensure good productivity (Soares et al., 2017). This constitutes a threat as these compounds easily reach the aquatic environment, being transported by rain or by direct application in the water as, for example, in rice plantations (Alho, 2008; Ross and Sanches, 2006). Crustaceans are a particularly sensitive group to the increasing chemical pressure in Pantanal. Studies in literature report higher sensitivity of shrimp species when compared with other aquatic species such as fish (Bajet et al., 2012). It is anticipated that this anthropogenic impact may lead to large-scale ecological imbalances, although there are still few studies on the toxicity of chemical pesticides to aquatic organisms living in this region (Dores, 2016).

One of the most used insecticides in the Pantanal region of Mato Grosso do Sul is cypermethrin (active ingredient of the formulation Barrage®), a synthetic pyrethroid used for agriculture, for household pest control and for fleas and ticks control in cattle (de Barros, 1992; Gomes et al., 2011; NPTN, 1998). The WWF estimates a herd of 22 million heads of cattle in the Pantanal, Upper Paraguay River Basin (WWF, 2018). According to indications of the manufacturer 5 ml of the product should be applied to each animal (diluted in a proper amount of water and delivered by spraying) for parasites control which would account for a total of 16500 Kg of cypermethrin per application (several are needed along the year) just for this specific usage.

In addition, this pesticide is used in aquacultures to control vectors in aquatic environments (Das and Mukherjee, 2003; Montanha, FP, 2012). Some European countries use 5 to 15 µg/L cypermethrin for 1 hour to control sea lice *Lepeophtheirus salmonis* in salmon hatcheries, releasing resulting waste water into the sea (Gowland et al., 2002; Hart et al., 1997). Similar procedures are observed in Brazil for the control of parasites in shrimp and fish aquaculture (Luvizotto-Santos et al., 2009; Martins, 2004). Cypermethrin acts as a neurotoxin that affects the central nervous system, being highly toxic to bees, aquatic insects, crustaceans and fish (Keith and Walker, 1992; NPTN, 1998). A previous study using larvae of the Pantanal endemic shrimp *M. pantanalense* exposed to the cypermethrin-based formulation Barrage® reported a 96h-LC_{50} as low as 0.05 µg/L (Soares et al., 2017).

The inclusion of histopathologic parameters in toxicity tests can reveal structural and specific toxic effects on organs at sublethal level (Dutra et al., 2017). Therefore, histopathology has been used in toxicity assessments aiming to identify tissue damage in aquatic animals exposed to contaminants (Dutra et al., 2017; Miron et al., 2008). Exposure of crustaceans to cypermethrin may damage the gill structure, as observed in the crab *Paratelphusa jacquemontii* exposed to cypermethrin (Nurocombi) insecticide, with signs of epithelial lifting, edema, necrosis, secondary lamella fusion, and hemorrhage in the gills (Maharajan et al., 2015). Cypermethrin also changes the proteins and structure of gills (vacuolization and collapse of gill filaments, edema and necrosis of epithelial cells, and rupture of epithelial layer) in red swamp crayfish (*Procambarus clarkii*), thereby impairing their physiological functions (Wei and Yang, 2015).

Given their close contact with the surrounding environment and high permeability, gills are an organ very sensitive to contaminants, and thus particularly important in biomonitoring and impact assessments studies (Maharajan et al., 2015; Wei and Yang, 2015). Furthermore, given that cypermethrin is a lipophilic pyrethroid with high affinity and solubility in lipids, this compound is highly absorbed by the gills (Polat et al., 2002). The hypothesis of this study is that cypermethrin, through the formulation Barrage®, can cause histopathological changes in gills of the Pantanal endemic shrimp *M. pantanalense.* The sensitivity of these organisms was also assessed through evaluation of mortality and swimming behavior (equilibrium and position).

2. Materials and methods

The study was carried out at the Laboratory of Carcinology, Shrimp Farming and Ornamental Organisms of Cerrado Pantanal (CARCIPANTA), located at the state of Mato Grosso do Sul, Brazil. Histological analyzes were performed at the Laboratory of

Experimental Pathology, Institute of Biosciences, located at the Federal University of Mato Grosso do Sul, Campo Grande, Brazil.

2.1 Chemicals

Cypermethrin ([α-cyano-3-phenoxybenzyl ester of 2,2-dimethyl-3-(2-2 dichlorovynil) cyclopropane carboxylic acid]; C_{22} H_{19} Cl_2 NO_3; CAS Number: 52315-07-8) source was the commercial formulation Barrage®, bought from Zoetis-Fort Dodge (Campinas, SP, Brazil). The formulation Barrage® is a concentrated suspension, emulsifiable, containing 150 g/L of cypermethrin. The stock solution was prepared by diluting the compound in fresh water (tap water) (28 ± 1°C, conductivity 0.24 µS/cm, pH 7.5 ± 0.5, and dissolved oxygen above 8 mg/L).

2.2 Toxicity test

M. pantanalense specimens were collected in Lagoa Baiazinha (latitude: 20º15'49"S and longitude: 56º23'11"W), a pristine place at Pantanal of Mato Grosso do Sul and kept in a closed, recirculating system, under controlled conditions: temperature of 28 ± 1 °C, conductivity of 0.24 µS/cm, pH 7.5 ± 0.5, dissolved oxygen above 8 mg/L and 12 h:12 h photoperiod cycle (light:dark). The shrimps were fed twice a day with adjusted diet (dry basis), containing 30% of crude protein and 4200 kcal/kg of gross energy and fish fillet, following the common laboratory procedure. Organisms were acclimatized in the lab for 1 week before the experiment and during this time no mortality, anomalous behavior or morphology were observed. For the toxicity assay 150 adults were weighed and measured from the tip of the rostrum to the tip of the telson using a digital caliper (Digimess®, 0-150mm). Shrimp weight and total length were, on average, 0.49 g (standard deviation of 0.17) and 40.17 mm (standard deviation of 4.33), respectively. A completely randomized design with six treatments (0, 0.05, 0.25, 1.25, 3.75 and 6.25 µg/L of cypermethrin) was used. Exposure solutions were prepared by successive dilution of the stock in culture medium. In each treatment 5 replicates with 5 shrimp each were used. The toxicity test was performed in glass tanks with aeration and 2.5 L of test solution. The test solution was renewed daily after feeding the shrimp. The criterion used to evaluate the mortality was the lack of response to mechanical stimulus by touching the shrimp with a glass rod. Shrimp were observed every 24 h until the end of 96 h to evaluate mortality, swimming behavior (normal swimming vs. swimming with equilibrium disturbances) position (normal position vs. side-lying). After 96 h, the live shrimp were collected for histological analyzes.

2.3 Histopathology of gills

Shrimp were euthanized by immersion in a tank containing approximately equal amounts of ice and water until the animals lose the ability to swim and the reflexes. Rapid cooling is a quick method that does not cause stress nor induces histological changes (Wilson et al., 2009). The cephalothorax of 3 shrimps per replicate were sectioned, grouped and fixed in 10% buffered formaldehyde (pH 6.84) for 24 hours. Subsequently, the gills were removed from the cephalothorax and fixed in 70% alcohol to be used for histological analyzes.

For the preparation of the histological slides, the gills were placed in cassettes fitted with filter paper, dehydrated in a graduated alcohol series (70%, 80%, 90% and absolute), cleared in three sequences of xylene and embedded in paraffin at 56 °C for 10 minutes each. After obtaining the paraplast-embedded blocks, tissues were sectioned at 5 µm thick sections using a rotary microtome (micron HM325) and then stained with hematoxylin-eosin. Histological images of the gills were captured using a microscope (Olympus BX41) at 10x and 40x magnification.

2.3.1 Qualitative and semi-quantitative analysis of the gills

Changes in gills were evaluated according to the histopathological condition indices for gills adapted from Bernet et al. (1999) and other related studies (Dutra et al., 2017; Rodrigues et al., 2017). Histopathological changes were classified into 5 categories: circulatory, regressive, progressive, inflammatory and neoplastic reaction patterns. The observed pathological change was ranked as a "Factor of importance", being classified as 1, 2 or 3, corresponding to the minimum (reversible pathological lesions), moderate (reversible lesions in most cases after the neutralization of the stressor agent) and severe (often irreversible lesions that cause partial or total loss of the function of the affected organ) pathological importance, respectively (**Table 1**). Each change was also assessed using a "score value" ranging from 1 to 6 (mild to severe occurrence) depending on its extent (i.e. percentage of areas in the gills exhibiting a specific alteration). From the classifications above, the Organ Index (Org I) was calculated according to the following equation: Org I = Σ_{cha} (a × w), where: "Org I" = Organ Index; "Cha" = change; "A" = score value; and "w" = factor of importance.

Table 1. Descriptions of histopathological categories and examples of specific changes assigned to each category for gills in the present study.

Histopathological categories	General description	Examples of specific tissue changes
		Gills
Circulatory	Disorders result from a pathological condition of the blood flow and tissue fluid	Intercellular edema (1)
Regressive	Disruption of tissue and/or cells that result in a functional reduction or loss of an organ. Changes in tissue architecture	Epithelial lifting of the lamellae (1) Lamellar fusion (1) Fat vacuoles (1) Nuclear changes (2) Atrophy (reduction of volume and number) (2) Necrosis (3)
Progressive	Increase in the number of cell types or specific structures	Hypertrophy of gill epithelial cells or mucous cells (1) Hyperplasia of gill epithelia (2)
Inflammatory	Presence of a greater number of cells involved in tissue repair; response to damaged tissue	Not observed
Neoplastic	Uncontrolled proliferation of cells and tissues	Not observed

( ) Factor of importance attributed to specific changes in gills

2.4 Statistical analysis

Statistical analysis was performed using the software SigmaPlot (version 12.5, Systat Software Inc., CA, USA) (Systat Software, 2014). Normality was verified using the Shapiro-Wilk normality test and the means were submitted to a one-way ANOVA. For non-normally distributed data, the nonparametric Kruskal-Wallis test was used. Means were compared by the Dunnett's test. The significance level for all statistical analyses was 0.05. The calculation of the LC_{50} values (lethal concentrations) was performed using the probit analysis in Minitab 17 Statistical Software (2010), with a 95% confidence interval.

3. Results

3.1 Acute test in adults of *M. pantanalense*

The effects of cypermethrin on the survival of adult shrimp *M. pantanalense* are shown in Fig. 1 (response curves) and Table 2 (LC_{50} calculation). The LC_{50} ranged from 2.75 µg/L (24 h of exposure) to 0.93 µg/L (96 h of exposure).

Table 2- LC_{50} values, the respective standard error and confidence interval for adults of *M. pantanalense*. Cypermethrin was used as the commercial formulation Barrage®.

Hours	LC_{50} (µg/L)	Error	95% confidence interval	
			Lower limit	Upper limit
24	2.75	0.27	2.24	3.38
48	2.11	0.24	1.68	2.67
72	1.67	0.21	1.29	2.17
96	0.92	0.12	0.71	1.25

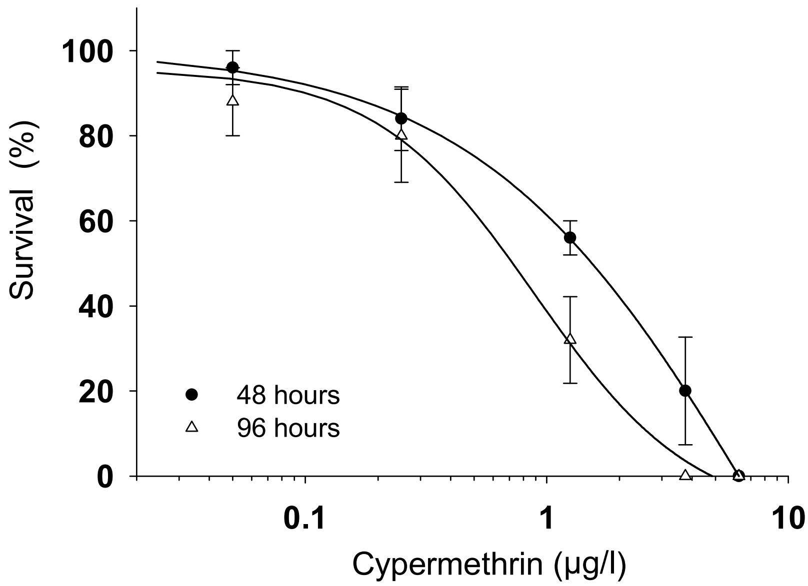

Figure 1. Cypermethrin effects in adults of *M. pantanalense*: survival after 48 and 96 h of exposure. Values represent means and error bars represent standard errors. The curve adjustment model was the four-parameter log-logistic function.

The effects of the compound observed in the behavior of adult shrimp *M. pantanalense* are shown in Fig. 2. For the highest concentrations of cypermethrin (1.25, 3.75 and 6.25 µg/L), shrimp showed signs of lack of equilibrium as they did not swim in a straight motion and could not assume an upright position (significant after 48 h of exposure to 1.25 µg/L cypermethrin; Kruskal-Wallis, H=20.29, P=0.001; **Fig. 2A**). It was also observed that shrimps tended to remain closer to the air stone of the tank. At the same concentrations, shrimp, when not in motion, were unable to be in an upright position laying in their side with spasmodic movements of the pereiopods (**Fig. 2B**), also significantly after 48 h of exposure to 1.25 μg/L (Kruskal-Wallis, H=20.73, P<0.001).

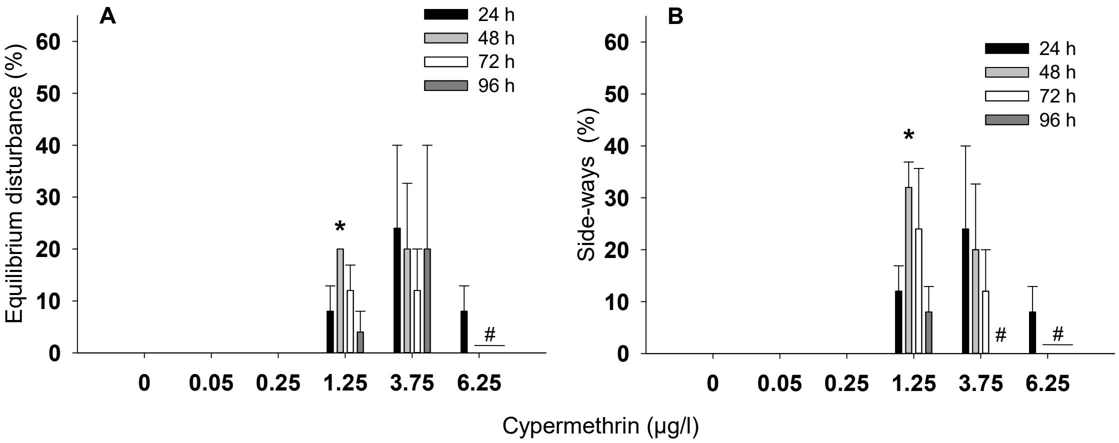

Figure 2. Cypermethrin effects in adults of *M. pantanalense*: Equilibrium disturbance (A); side-ways (B). Values represent means and error bars represent standard errors. * denote statistically significant differences relative to the control ($p < 0.05$)."#" indicates mortality.

3.2 Histopathological analysis of the gills

3.2.1 Qualitative analysis

The effects of Barrage® on the gills of adult shrimp *M. pantanalense* are shown in Figure 3. Individuals of the control group exhibited normal structure of gill filaments during the exposure period (Fig. 3A), with few cases of intercellular edema and mucous cell hyperplasia (score 2, 21-30%). Progressive lesions such as hyperplasia of mucosal cells and epithelial cells (Fig. 3D) were observed in shrimp exposed to concentrations 0.05, 0.25 and 1.25 μg/L of cypermethrin. Hypertrophy of mucosal cells was also observed in the gills of shrimp exposed to concentrations 0.25 and 1.25 μg/L. Several regression lesions were observed in the gills of shrimp exposed to concentrations 0.05, 0.25 and 1.25 μg/L, including alterations in epithelial structure, epithelial lifting of the lamellae, and changes in tissue structure such as shortening of secondary lamellae (Fig. 3B and C), lamellar fusion (Fig. 3B), as well as fat vacuoles, nuclear changes and atrophy when exposed to 0.25 and 1.25 μg/L Barrage®. At the concentration 1.25 μg/L, shrimp also exhibited necrosis. Circulatory alterations such as the presence of edema were also observed at 0.05, 0.25 and 1.25 μg/L (Fig. 3C and D). Inflammatory and neoplastic changes were not found.

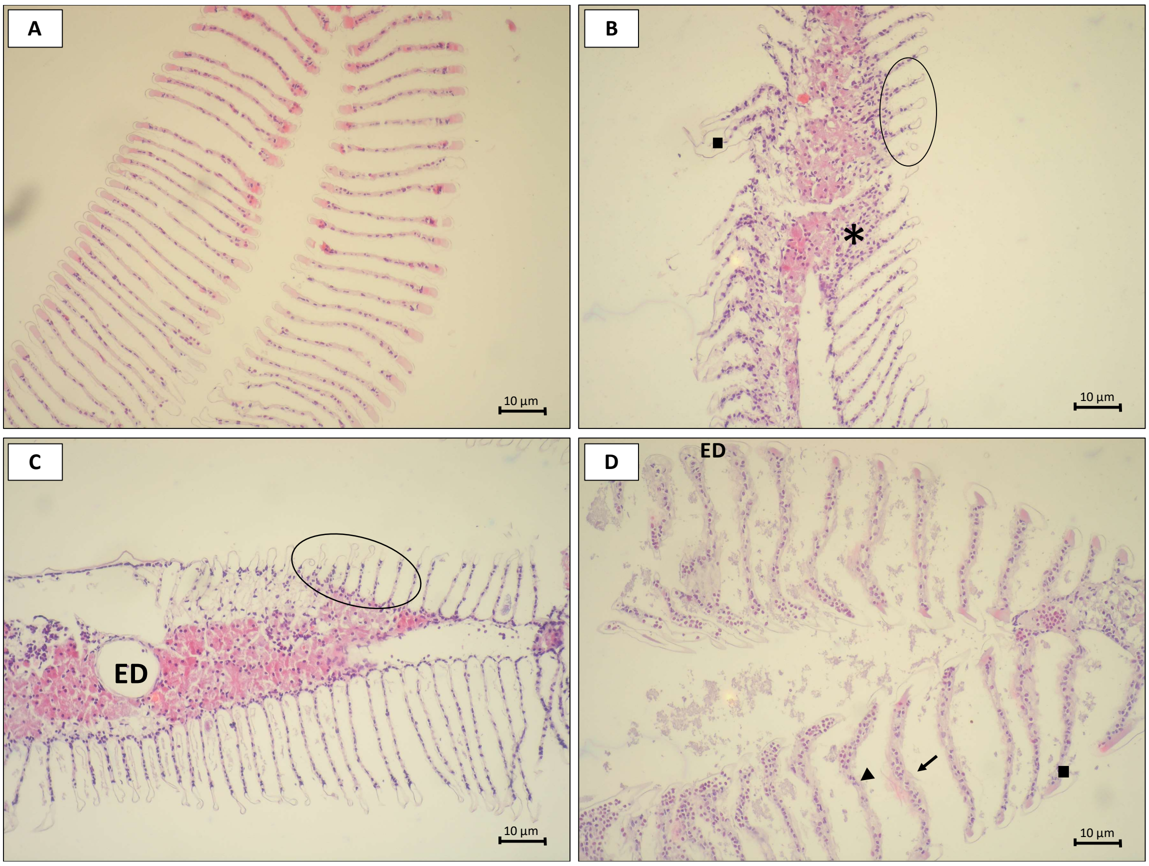

Figure 3. Histological sections of the gills from *M. pantanalense* after acute exposure to cypermethrin through the formulation Barrage®. Photomicrographs of histological section of gill filaments of control (A) and organisms exposed to 0.05 μg/L (B), 0.25 μg/L (C) and 1.25 μg/L (D); Lamellar fusion (*), shortening of secondary lamellae (solid circle), Edema (ED), Hyperplasia of epithelial cells and mucous cells (black arrow), Epithelial lifting of the lamellae (black square), Nuclear changes (black triangle), Hematoxylin and eosin stain; 10 times magnification.

3.2.1 Semi-quantitative analysis

After acute exposure, regression alterations were the most predominant in the gills, as shown in Fig. 4; the lesions were significantly increased at 0.25 and 1.25 μg/L (One-way ANOVA, F=7.52; $p<0.01$). Despite an increase in lesions, no significant change was observed in the circulatory (One-way ANOVA, F=1.88, p=0.21) and progressive categories (One-way ANOVA, F=2.38, p=0.14). Overall, organisms exposed to concentrations of 0.25 and 1.25 μg/L showed increased total pathological indices for gills relative to the control (One-way ANOVA, F=29.46, $p<0.001$) after the exposure period of 96 h (Fig. 4).

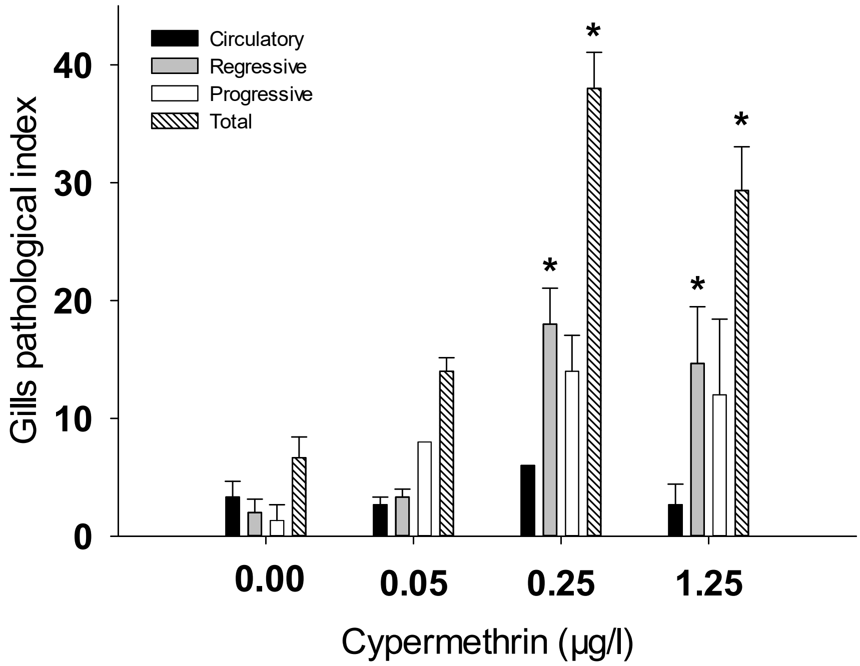

Figure 4. Total pathological condition indices and categories (circulatory, regressive and progressive) for gills from *M. pantanalense* shrimp after acute exposure to cypermethrin. Values represent means of each treatment ± standard error. * denotes condition indices significantly different relative to the control (Dunnett's test, $p < 0.05$).

4. Discussion

The objective of this study was to evaluate the effects of the formulation Barrage®, a cypermethrin-based pesticide widely used in Pantanal, on adults of the endemic shrimp *M. pantanalense*. Histopathological effects have been studied to elucidate how the formulation can affect gill structure of shrimp at sublethal levels.

The 96h-LC_{50} value found for adult *M. pantanalense* (0.93 µg/L) was higher than the previously reported for larvae of this species using the same formulation of Barrage® (0.05 µg/L) (Soares et al., 2017). This result is not surprising given that during larval development the central nervous system is still forming conferring higher susceptibility of larvae towards neurotoxic compounds (Anger, 2001; Arnberg et al., 2013). The 96h-LC_{50} value observed was also higher than other values reported in the literature for adult shrimps, such as 0.02 µg/L for *Palaemonetes pugio* (using pure cypermethrin) (DeLorenzo et al., 2014), 0.019 µg/L for the freshwater prawn *Paratya* *australiensis* (pure cypermethrin) (Kumar et al., 2010), 0.11 μg/L for *Penaeu duorarum* (pure cypermethrin) (Cripe, 1994) and 0.002 μg/L for juveniles of *Palaemonetes argentines* (cypermethrin based formulation Sherpa®) (Collins and Cappello, 2006).

Besides different intrinsic species sensitivities, differences obtained may also be explained by the tested compound. The use of formulations, although representing a more realistic exposure scenario, may not translate the toxicity of the pure compound as the presence of additional constituents in their composition can add to or modify the toxicity of the active compound. In general, the high toxicity of cypermethrin-based formulations to crustaceans is not surprising since these compounds, like other pyrethroids, have been designed to control arthropod pests. Environmental factors such as salinity may also play a role on the toxicity of the chemical compounds as shown by Wang et al (2013) in a study where the 96h-LC_{50} value for the shrimp *Litopenaeus vannamei* exposed to cypermethrin varied from 0.17 to 0.38 μg/L at salinity 5 and 20 % respectively.

High concentrations of Cypermethrin (1.25 and 3.75 μg/L) led to swimming difficulties apparently caused by equilibrium disturbances, inability to keep an upright position and spasmodic movements of pereiopods of shrimps. Information on the toxicity of this formulation to shrimps is limited. Comparable effects were observed using Excis®, a cypermethrin-based formulation, in adult lobsters *Homarus americanos*, which showed signs of lethargy, uncoordinated movements, claws either crossed or extended laterally and inactivity with spasmodic movements of pereiopods; the estimated 48h-LC_{50} was 0.08 μg/L (Burridge et al., 2000). These symptoms suggest an impairment of the motor function caused by neuronal disturbance (Keith and Walker, 1992; NPTN, 1998). Behavior disruption may have important consequences at population level as it may translate in impairment of important functions as feeding, predator avoidance and reproduction.

In addition to the above-mentioned effects, shrimp swam near the air stone of the tank in search of oxygen probably because cypermethrin increases oxygen consumption due to an acceleration of metabolism, as observed in juveniles of *Palaemonetes argentinus* (Collins and Cappello, 2006). The authors observed that shrimp exposed to 0.0002, 0.0025 and 0.025 μg/L of cypermethrin (through the formulation Sherpa) for 96 hours increased hyperactivity, as well as oxygen uptake and nitrogen excretion measured as ammonia-N.

The gill is a multi-functional organ particularly important to crustaceans since it represent the fundamental site for gas exchange, regulation of ions, excretion of metabolic products, and then, a potential target for contaminants (Wei and Yang, 2015). The formulation Barrage® induced significant histopathological changes in gills of Pantanal shrimp *M. pantanalense* during acute exposure, with regressive lesions predominating (at 0.25 and 1.25 µg/L of cypermethrin). Although no significant difference was observed, an increase in progressive lesions was also recorded with increasing cypermethrin concentrations. A list of histological findings in the gills of different aquatic organisms (crustaceans and fish) exposed to cypermethrin is presented in Table 3. Overall, histological changes similar to those observed in the present study, such as intercellular edema, epithelial lifting of the lamellae, lamellar fusion, hypertrophy of gill epithelial cells or mucosal cells, hyperplasia of gill epithelia and necrosis have already been observed in previous studies with aquatic organisms exposed to cypermethrin (Arslan et al., 2017; Korkmaz et al., 2009; Moraes, 2013; Velisek et al., 2006; Velmurugan et al., 2009; Wei and Yang, 2015). On the other hand, changes in the gill structure, such as fat vacuoles, nuclear changes and atrophy (reduction of volume and number) observed in this study with Pantanal shrimp were not reported as effects of cypermethrin in previous studies. Given that pesticide formulations contain a mixture of chemicals (Elhalwagy and Zaki, 2009), these effects may be related to other unidentified compounds in the blend such as C8, C9 and C10 aromatic compounds, which were already detected in the formulation Barrage® (Soares et al., 2017) and other pyrethroid-based formulations (Magdalan et al., 2009).

Pyrethroids are lipophilic and have a high absorption rate by the gills (Polat et al., 2002), directly affecting their structures. Lesions such as epithelial lifting, lamellar fusion, shortening of secondary lamellae, hypertrophy and hyperplasia of epithelia can be understood as a protective mechanism of shrimp, reducing the contact surface of the gills with the contaminated fluid and thus the contact with the hemolymph (Çaliskan et al., 2003; Maharajan et al., 2015). This mechanism can, however, affect gas exchange across the gills (Korkmaz et al., 2009). Necrosis is a more invasive deleterious consequence (Maharajan et al., 2015). Histopathological changes can compromise physiological processes and lead to hypoxia, respiratory failure and even death of organisms (Çaliskan et al., 2003). The histopathological condition index indicated greater effects of the formulation Barrage® on regressive alterations, classified as reversible lesions if the contaminant was eliminated from the medium (Bernet et al., 1999).

Taking into account the widespread use of Barrage® in Pantanal for agricultural livestock and domestic purposes, a continuous input of residues in the environment is expectable (de Barros, 1992; Gomes et al., 2011; NPTN, 1998). Thus, although concentrations tested in this study are not plausible to be found in the environment, this study highlights the need of refining the assessment of this pesticide in relevant exposure scenarios (*e.g.* lower concentrations) and calls the attention for the need of proper environmental monitoring and assessment of risk of pesticides use in Pantanal.

Table 3. Review of cypermethrin effects on gills of different species of crustaceans and fish.

	Species	Chemical	Concentration	Changes	Reference
Fish	*Cyprinus carpio*	Cypermethrin	0.01 and 0.005 ppm	Hyperplasia of lamellar cells; telangiectasia of lamellae and thickening due to inflammatory cells infiltration.	Arslan et al., 2017
	Brycon amazonicus	Cypermethrin (Galgotrin)	7.2 µg/L	I-Hypertrophy and hyperplasia of chloride cells, vasodilation and apical aneurysm. II - Aneurysm and hemorrhage with rupture of the lamellar epithelium.	Moraes 2013
	Oreochromis niloticus	Cypermethrin	0.44 µg/L	Edema and hypertrophy of epithelial cells. Epithelial hyperplasia, necrosis, desquamation, fusion of secondary lamellae and 'curling' of secondary lamellae.	Korkmaz et al., 2009
	Clarias gariepinus	Cypermethrin	10.05, 20.10 and 30.15 µg/L	Epithelial hypertrophy, epithelial lifting and edema; hyperplasia of primary epithelial cells, fusion of secondary lamellae and necrosis and desquamation.	Velmurugan et al., 2009
	Oncorhynchus mykiss	Cypermethrin (Alimetrine)	31.4 µg/L	Severe telangiectasia of secondary gill lamellae with the rupture of pillar cells.	Velisek et al., 2007
	Lebistes reticulatus	Zeta-cypermethrin	20, 26, 35 µg/L	Lifting of epithelial layer and necrosis. Exudation, hyperplasia and the shortening of secondary lamellae.	Çaliskan et al., 2003
Crustacean	*Procambarus clarkii*	Beta-cypermethrin	0.005, 0.01 and 0.04 µg/L	Gill filaments were swollen, and lamellar epithelial cells appeared to be fused or necrotic. Gill lamellae exhibited peculiar malformations.	Wei and Yang 2015
	Paratelphusa jacquemontii	Cypermethrin (Nurocombi)	0.018 and 0.037 mg/L	Enlargement of interlamellar space densely packed with granular	Maharajan et al., 2015

			material, and loss of gill structure; the gill lamellae get collapsed due to the disruption of the pillar cells; hemocoel filled with coarse amorphous to fibrous materials, thickened gill lamellae, and massive hemocytic infiltration; detached cuticle and rupture of capillaries at tip of the secondary lamellae releasing haemocytes; bulbular swelling at the tip; epithelial necrosis and hyperplasia; enlargement and disarrangement of secondary gill lamellae and lamellar fusion in some regions.	
Macrobrachium pantanalense	Cypermethrin (Barrage®)	0.05, 0.25 and 1.25 µg/L	I- Intercellular edema, epithelial lifting of the lamellae, lamellar fusion, fat vacuoles and hypertrophy of gill epithelial cells or mucous cells. II- Nuclear changes, atrophy (reduction of volume and number) and hyperplasia of gill epithelia. III- necrosis.	**Present study**

5. Conclusion

The formulation Barrage®, a cypermethrin-based pesticide widely used in the region of Pantanal (Brazil) was reported to cause behavioral and histological effects in the endemic shrimp *M. pantanalense*. Behavioral effects were characterized by equilibrium disruption and lethargy, translated in swimming difficulties and hypoactivity and denote neuronal effects of the active compound, cypermethrin. Histopathological effects included relevant structural lesions in gills which may affect their physiological function. Given the lack of knowledge on environmental concentrations of Barrage® in the Pantanal, this study calls the attention for the need of performing long term studies using environmental concentrations on local species to accurately assess impact of Barrage® in the aquatic communities of this fragile Biome. Given the increasing economic activity in the region population awareness of the risks of pesticide use, education for the correct use of pesticides as well as better policies for environmental protection are also needed.

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6. References

Alho, C.J.R., 2008. Biodiversity of the Pantanal: response to seasonal flooding regime and to environmental degradation. Braz. J. Biol. 68, 957–966. doi:10.1590/S1519-69842008000500005

Anger, K., 2001. The Biology of Decapod Crustacean Larvae, 14th ed. Lisse: AA Balkema Publishers.

Arnberg, M., Calosi, P., Spicer, J.I., Tandberg, A.H.S., Nilsen, M., Westerlund, S., Bechmann, R.K., 2013. Elevated temperature elicits greater effects than decreased pH on the development, feeding and metabolism of northern shrimp (*Pandalus borealis*) larvae. Mar. Biol. 160, 2037–2048. doi:10.1007/s00227-012-2072-9

Arslan, H., Özdemir, S., Altun, S., 2017. Cypermethrin toxication leads to histopathological lesions and induces inflammation and apoptosis in common carp (*Cyprinus carpio* L.). Chemosphere 180, 491–499. doi:10.1016/j.chemosphere.2017.04.057

Bajet, C.M., Kumar, a., Calingacion, M.N., Narvacan, T.C., 2012. Toxicological assessment of pesticides used in the Pagsanjan-Lumban catchment to selected non-target aquatic organisms in Laguna Lake, Philippines. Agric. Water Manag. 106, 42–49. doi:10.1016/j.agwat.2012.01.009

Bernet, D., Schmidt, H., Meier, W., Burkhardt-Holm, P., Wahli, T., 1999. Histopathology in fish: proposal for a protocol to assess aquatic pollution. J. Fish Dis. 22, 25–34. doi:10.1046/j.1365-2761.1999.00134.x

Burridge, L.., Haya, K., Waddy, S.., Wade, J., 2000. The lethality of anti-sea lice formulations Salmosan® (Azamethiphos) and Excis® (Cypermethrin) to stage IV and adult lobsters (*Homarus americanus*) during repeated short-term exposures. Aquaculture 182, 27–35. doi:10.1016/S0044-8486(99)00251-3

Çaliskan, M., Erkmen, B., Yerli, S. V., 2003. The effects of zeta cypermethrin on the gills of common guppy *Lebistes reticulatus*. Environ. Toxicol. Pharmacol. 14, 117–120. doi:10.1016/S1382-6689(03)00046-2

Collins, P., Cappello, S., 2006. Cypermethrin toxicity to aquatic life: Bioassays for the freshwater prawn *Palaemonetes argentinus*. Arch. Environ. Contam. Toxicol. 51, 79–85. doi:10.1007/s00244-005-0072-1

Cripe, G.M., 1994. Comparative acute toxicities of several pesticides and metals to *Mysidopsis bahia* and postlarval *Penaeus duorarum*. Environ. Toxicol. Chem. 13, 1867–1872. doi:10.1002/etc.5620131119

Das, B.K., Mukherjee, S.C., 2003. Toxicity of cypermethrin in *Labeo rohita* fingerlings: biochemical, enzymatic and haematological consequences. Comp. Biochem. Physiol. Part C Toxicol. Pharmacol. 134, 109–121. doi:10.1016/S1532-0456(02)00219-3

de Barros, A.T.M., 1992. Recomendações para controle da mosca-dos-chifres no Pantanal. ainfo.cnptia.embrapa.br.

De Grave, S., Cai, Y., Anker, a., 2008. Global diversity of shrimps (Crustacea: Decapoda: Caridea) in freshwater. Hydrobiologia 595, 287–293. doi:10.1007/s10750-007-9024-2

DeLorenzo, M.E., Key, P.B., Chung, K.W., Sapozhnikova, Y., Fulton, M.H., 2014. Comparative toxicity of pyrethroid insecticides to two estuarine crustacean species, *Americamysis bahia* and *Palaemonetes pugio*. Environ. Toxicol. 29, 1099–1106. doi:10.1002/tox.21840

Dores, E.F.G. de C., 2016. Pesticides in the Pantanal, in: Bergier, I., Assine, M.L. (Eds.), Dynamics of the Pantanal Wetland in South America. Switzerland: Springer International Publishing, pp. 179–190. doi:10.1007/978-3-319-18735-8

Dutra, F.M., Ronnau, M., Sponchiado, D., Forneck, S.C., Ballester, C.A.F., Cupertino, E.L., 2017. Histological alterations in gills of *Macrobrachium amazonicum* juveniles exposed to ammonia and nitrite. Aquat. Toxicol. 187, 115–123. doi:10.1016/j.aquatox.2017.04.003

Elhalwagy, M., Zaki, N., 2009. Comparative study on pesticide mixture of organophosphorus and pyrethroid in commercial formulation. Environ. Toxicol. Pharmacol. 28, 219–224. doi:https://doi.org/10.1016/j.etap.2009.04.007

Gomes, A., Koller, W., Barros, A., 2011. Susceptibility of *Rhipicephalus* (*Boophilus*) *microplus* to acaricides in Mato Grosso do Sul, Brazil. Ciência Rural 41, 1447–1452. doi:http://dx.doi.org/10.1590/S0103-84782011005000105

Gowland, B.T.., Moffat, C.F., Stagg, R.M., Houlihan, D.F., Davies, I.M., 2002. Cypermethrin induces glutathione S-transferase activity in the shore crab, *Carcinus maenas*. Mar. Environ. Res. 54, 169–177. doi:10.1016/S0141-1136(02)00105-8

Hart, J.L., Thacker, J.R., Braidwood, J.C., Fraser, N.R., Matthews, J.E., 1997. Novel cypermethrin formulation for the control of sea lice on salmon (*Salmo salar*). Vet. Rec. 140, 179–81. doi:10.1136/VR.140.7.179

Junk, W.J., da Cunha, C.N., Wantzen, K.M., Petermann, P., Strüssmann, C., Marques, M.I., Adis, J., 2006. Biodiversity and its conservation in the Pantanal of Mato Grosso, Brazil. Aquat. Sci. 68, 278–309. doi:10.1007/s00027-006-0851-4

Keith, L.H., Walker, M., 1992. EPA'S Pesticide Fact Sheet Database.

Korkmaz, N., Cengiz, E.I., Unlu, E., Uysal, E., Yanar, M., 2009. Cypermethrin-induced histopathological and biochemical changes in Nile tilapia (*Oreochromis niloticus*), and the protective and recuperative effect of ascorbic acid. Environ. Toxicol. Pharmacol. 28, 198–205. doi:10.1016/j.etap.2009.04.004

Kumar, a., Correll, R., Grocke, S., Bajet, C., 2010. Toxicity of selected pesticides to freshwater shrimp, *Paratya australiensis* (Decapoda: Atyidae): Use of time series acute toxicity data to predict chronic lethality. Ecotoxicol. Environ. Saf. 73, 360–369. doi:10.1016/j.ecoenv.2009.09.001

Luvizotto-Santos, R., Eler, M.N., Espindola, E.L.G., Vieira, E.M., 2009. O uso de praguicidas nas pisciculturas e pesqueiros situados na Bacia do rio Mogi-Guaçu. Bol. do Inst. Pesca 35, 343–358.

Magdalan, J., Zawadzki, M., Merwid-Lad, A., 2009. Fatal intoxication with hydrocarbons in deltamethrin preparation. Hum. Exp. Toxicol. 28, 791–793. doi:https://doi.org/10.1177/0960327109354939

Maharajan, A., Narayanasamy, Y., Ganapiriya, V., Shanmugavel, K., 2015. Histological alterations of a combination of Chlorpyrifos and Cypermethrin (Nurocombi) insecticide in the fresh water crab, *Paratelphusa jacquemontii* (*Rathbun*). J. Basic Appl. Zool. 72, 104–112. doi:10.1016/j.jobaz.2015.08.002

Martins, M.L., 2004. Cuidados Básicos e Alternativas no Tratamento de Enfermidades de Peixes na Aqüicultura Brasileira, in: Editora Varela (Ed.), Sanidade de Organismos Aquáticos. pp. 355–368.

Melo, G.A.S., 2003. Manual de identificação dos Crustácea Decápoda de água doce do Brasil. São Paulo: Editora Loyola.

Miron, D. dos S., Moraes, B., Becker, A.G., Crestani, M., Spanevello, R., Loro, V.L., Baldisserotto, B., 2008. Ammonia and pH effects on some metabolic parameters and gill histology of silver catfish, *Rhamdia quelen* (*Heptapteridae*). Aquaculture 277, 192–196. doi:10.1016/J.AQUACULTURE.2008.02.023

Montanha, FP, C.P., 2012. Efeitos toxicológicos de piretróides (cipermetrina e deltametrina) em peixes-Revisão. Rev. Científica Eletrônica Med. Veterinária 18, 1–58.

Moraes, F.D. de, 2013. Respostas bioquímicas, genotóxicas, fisiológicas e histológicas de matrinxã (*Brycon amazonicus*, Spix; Agassiz 1829) exposto à cipermetrina (Galgotrin®). Universidade Federal de São Carlos.

Murphy, N.P., Austin, C.M., 2005. Phylogenetic relationships of the globally distributed freshwater prawn genus *Macrobrachium* (Crustacea: Decapoda: *Palaemonidae*): Biogeography, taxonomy and the convergent evolution of abbreviated larval development. Zool. Scr. 34, 187–197. doi:10.1111/j.1463-6409.2005.00185.x

NPTN, 1998. National Pesticide Information Center -Cypermethrin. Oregon.

Polat, H., Erkoç, F.Ü., Viran, R., Koçak, O., 2002. Investigation of acute toxicity of beta-cypermethrin on guppies *Poecilia reticulata*. Chemosphere 49, 39–44. doi:10.1016/S0045-6535(02)00171-6

Rodrigues, S., Antunes, S.C., Nunes, B., Correia, A.T., 2017. Histological alterations in gills and liver of rainbow trout (*Oncorhynchus mykiss*) after exposure to the antibiotic oxytetracycline. Environ. Toxicol. Pharmacol. 53, 164–176. doi:10.1016/J.ETAP.2017.05.012

Ross, J., Sanches, L., 2006. Plano de conservação da bacia do alto Paraguai e o zoneamento ecológico-econômico para o Brasil. An. 1º Simpósio Geotecnologias no Pantanal, Campo Gd. Bras. 1, 11–15.

Santos, A.D.O.S., Hayd, L., Anger, K., 2013. A new species of *Macrobrachium* Spence Bate, 1868 (Decapoda, Palaemonidae), *M. pantanalense*, from the Pantanal, Brazil 3700, 534–546.

Soares, M.P., Jesus, F., Almeida, A.R., Zlabek, V., Grabic, R., Domingues, I., Hayd, L., 2017. Endemic shrimp *Macrobrachium pantanalense* as a test species to assess potential contamination by pesticides in Pantanal (Brazil). Chemosphere 168, 1082–1092. doi:10.1016/j.chemosphere.2016.10.100

Minitab 17 Statistical Software (2010). [Computer software]. State College, PA: Minitab, Inc. (www.minitab.com).

Systat Software, I., 2014. SigmaPlot for Windows Version 12.5 G, Germany.

Velisek, J., Wlasow, T., Gomulka, P., Svobodova, Z., Dobsikova, R., Novotny, L., Dudzik, M., 2006. Effects of cypermethrin on rainbow trout (*Oncorhynchus mykiss*). Vet. Med. 51, 469–476.

Velmurugan, B., Mathews, T., Cengiz, E.I., 2009. Histopathological effects of cypermethrin on gill, liver and kidney of fresh water fish *Clarias gariepinus* (Burchell, 1822), and recovery after exposure. Environ. Technol. 30, 1453–1460. doi:10.1080/09593330903207194

Wang, H.W., Wang, H., Zhao, C.L., Yuan, G.F., Ma, L., Li, L.F., Cai, D.B., 2014. Effects of Cypermethrin on the Superoxide Dismutase Activity of *Macrobrachium nipponense.* Adv. Mater. Res. 955–959, 554–557. doi:10.4028/www.scientific.net/AMR.955-959.554

Wang, X., Li, E., Xiong, Z., Chen, K., Yu, N., Du, Z., Chen, L., 2013. Low Salinity Decreases the Tolerance to Two Pesticides, Beta-cypermethrin and Acephate, of White-leg Shrimp, *Litopenaeus vannamei*. J. Aquac. Res. Dev. 4. doi:10.4172/2155-9546.1000190

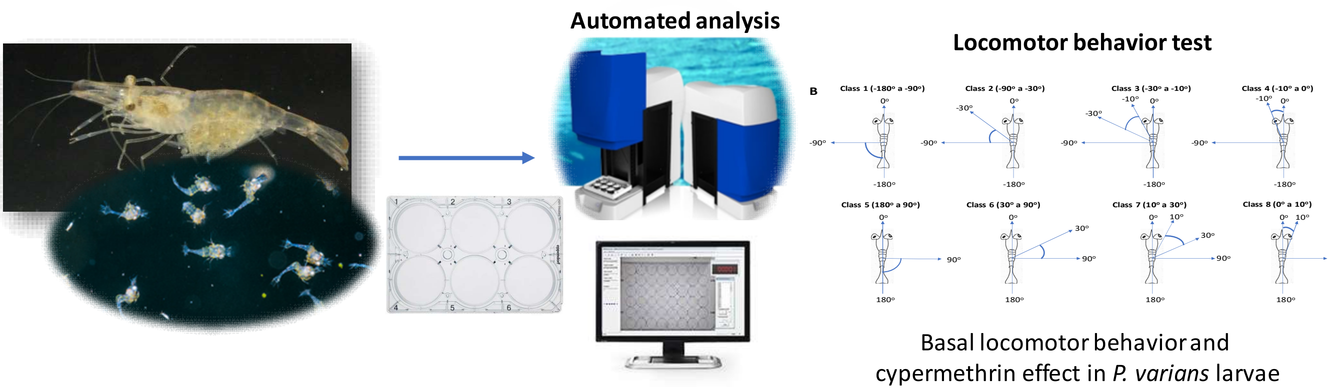
Wei, K., Yang, J., 2015. Oxidative damage induced by copper and beta-cypermethrin in gill of the freshwater crayfish *Procambarus clarkii*. Ecotoxicol. Environ. Saf. 113, 446–453. doi:10.1016/j.ecoenv.2014.12.032

Wilson, J.M., Bunte, R.M., Carty, A.J., 2009. Evaluation of Rapid Cooling and Tricaine Methanesulfonate (MS222) as Methods of Euthanasia in Zebrafish (*Danio rerio*). Am. Assoc. Lab. Anim. Sci. 48, 785–789.

WWF, 2018. Pecuária Sustentável [WWW Document]. WWF-World World Wide Fund for Nature Brazil. URL: https://www.wwf.org.br/natureza_brasileira/areas_prioritarias/pantanal/nossas_solucoes_no_pantanal/desenvolvimento_sustentavel_no_pantanal/pecuaria_sustentavel_no_pantanal/

Chapter 5

Barrage® behavioral effects in larval stages of *Palaemon varians* and *Danio rerio*



Barrage® behavioral effects in larval stages of *Palaemon varians* and *Danio rerio*

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Abstract

Behavior has been increasingly used in ecotoxicology due to its high sensitivity towards chemical compounds. This study aimed to develop methodologies using the automated video tracking system Zebrabox for behavioral analysis in larvae of the shrimp *Palaemon varians*. Moreover, effects of the cypermethrin-based pesticide Barrage® were assessed using larvae of *P. varians* (developmental and behavioral effects) and *Danio rerio* (behavioral effects). A sudden switch in light conditions (dark to light) constituted a stimulus (startle) that elicited an increased motor activity in *P. varians* larvae (hyperactivity and erratic swimming). However, the intensity of this response decreases with the repetition of the stimulus suggesting the adaptation of shrimp larvae. Exposure of shrimp larvae to Barrage® elicited developmental effects similar to the observed for other shrimp species (growth inhibition, decrease of lipid droplets and developmental delay) suggesting energy allocation to fight Barrage® toxicity. At behavioral level, larvae exposed to Barrage® showed a slower or incomplete recovery from the response to the startle when comparing to the control. This was observed at concentrations as low as 0.01 μg/L, confirming the high sensitivity of behavioral parameters and rising concerns on the risk of Barrage® residues in the aquatic environment for shrimp species. Zebrafish behavioral response to Barrage® was observed at concentrations from 156 μg/L and were characterized by hypoactivity and erratic swimming. This study reinforces the importance of behavior as an endpoint in risk assessment of chemical compounds and shows, through the Barrage® case-study, the potential of shrimp species as model organisms in behavior ecotoxicology.

Keywords: Cypermethrin; locomotion; shrimp, fish, neurotoxic compounds

1. Introduction

Behavior is a response at organism level defined as the action, reaction or functioning of a system under a set of specific circumstances, resulting from the integration of conditions to which organisms are exposed (Hellou, 2011). The behavioral response of an organism is mediated through several internal mechanisms (biochemical and physiological processes) which respond to environmental stimulus including stress factors and very often reflect changes at higher levels of organization with ecological relevance (Gerhardt, 2007). As an endpoint, it has been observed that behavioral responses are 10 to 1000 times more sensitive that lethality and thus, behaviour has been suggested as a useful tool for early detection of environmental risk (Hellou, 2011; Robinson, 2009). Behavioral ecotoxicology is, thus, an interdisciplinary field, involving ecology, ethology, and toxicology that aims to understand how exposure to contaminants can alter individual behavioral components and understand the consequences to the fitness of populations and communities (Dell'Omo, 2002).

In the last decades traditional rodent behavior methodologies have been successfully adopted for zebrafish (*Danio rerio*) that has been emerging as a good model for assessing effects of neuro-active drugs (Champagne et al., 2010). These tests generally consist in exposing the fish to a mildly stressful situation eliciting typical innate fear or anxiety-like behaviors, which can be assessed by using the exploratory capacity of the fish and measuring behavioral components such as avoidance, freezing, erratic swimming, hyperactivity, shoaling, among others (Maximino et al., 2010). Since automated tracking systems area available in the market allowing fast and accurate analysis of locomotor behavior, research in this area is growing, including the development of procedures for other types of organisms including crustaceans. In this work, methodologies used in zebrafish larvae using the video tracking system Zebrabox

(Viewpoint Life sciences, Lyon, France) will be adapted to larvae of the shrimp *Palaemon varians* to assess their suitability to behavioural analysis. *P. varians* is often used as model species in ecotoxicology, has a short larval development and easy identification (Jorge Palma et al., 2008; Cottin et al., 2012; Rainbow e Smith, 2013; New et al., 2014; Correia et al., 2016; Pavlaki et al., 2016). During larval development they go through five phases of zoea until reaching the decapodite phase (Fincham, 1979). In the larval stage they present active phototaxis, being attracted by light, using thoracic appendages for swimming in the water column upside down (Anger, 2001).

Shrimp species are very sensitive to chemical compounds and previous studies have already reported their usefulness for behavioral assessment of neurotoxic compounds effects. Previous works have reported, for example, the influence of sublethal concentrations of pesticides, such as methamidophos, on the behavior of Penile prawns (*Litopenaeus vannamei*), with hyperactivity, disordered swimming and spasms being considered as effects on the nervous system (García-de la Parra et al., 2006). Adult shrimps of the genus Palaemon (*P. serratus*) responded to anthracene by decreasing swimming speed (Gravato et al., 2014) while the same effects was also observed in adult shrimp of *P. serratus* exposed to Benzo (a) pyrene (Silva et al., 2013) and in post-larvae of *Penaeus monodon* exposed via feeding to mercury (Harayashiki et al., 2016).

The model compound used in the present work was the formulation Barrage®, a pesticide based on the pyrethroid cypermethrin widely used as a vector control in agriculture, cattle and in domestic residences for pest control (Barros, 1992; Gomes et al., 2011; NPTN, 1998). In addition, cypermethrin is used in aquaculture in European countries and also in Brazil for the control of parasites in fish (Geferson et al., 2015; Hart et al., 1997; Luvizotto-Santos et al., 2009; Martins, 2004; Montanha, FP, 2012; Tu et al., 2010). Cypermethrin causes damage to the central nervous system, acting as a potent neurotoxin to bees, microcrustaceans, crustaceans, and fish (Keith and Walker, 1992; NPTN, 1998). Toxicity values of 0.05 μg/L (96h LC_{50}) and 1680 μg/L (144h LC_{50}) of cypermethrin have been reported in previous work of the group for the larvae of the shrimp *Macrobrachium pantanalense* and *D. rerio* respectively (using the formulation Barrage®) (Soares et al., 2017).

The hypothesis of this study is that *P. varians* shrimp larvae can be used in behavioral assessments and that cypermethrin through the Barrage® formulation can disturb swimming behavior. The specific objectives of this study were i) to evaluate the basal locomotor behavior for larvae of *P. varians* using the automated video tracking system Zebrabox; ii) to determine the acute toxicity of Barrage® to *P. varians* larvae by assessing lethal and developmental effects; iii) to determine behavioral toxicity of sublethal concentrations of Barrage® to *P. varians* and *D. rerio* larvae.

2. Material and Methods

2.1 Chemicals

The source of cypermethrin (α-cyano-3-phenoxybenzyl-2,2-dimethyl-3- (2,2-dichlorovinyl) -cyclopropanecarboxylate); CAS number: 52315-07-8) was the commercial formulation Barrage® (Zoetis-Fort Dodge, Campinas, SP, Brazil). This formulation is a concentrated, emulsifiable suspension containing 150 g of cypermethrin per liter. The exposure solutions were prepared by dilution of the formulation in artificial sea water in the case of *P. varians* assays or in fish culture water in the case of zebrafish assays.

2.2.1. Shrimp larvae toxicity assay

Ovigerous females of *P. varians* were collected at the Salina da Troncalhada, Aveiro, Portugal (40º38'40.1"N, 8º39'52.0"W) and transported to the laboratory at the University of Aveiro. Organisms were individually transferred to 25 L aquariums and fed 4 times daily with commercial feed *ad libitum*. The aquariums contained a mobile plastic net, dividing them into two parts: one with shelters (pieces of PVC tube of 32 mm) that served as a refuge for ovate females, and another with a focused beam of LED light. Newly hatched larvae cross the net into the lit part due to positive phototaxis, preventing cannibalism by mothers. Shrimps were maintained in artificial seawater (Sea salt Ocean fish, PRODAC International, Italy) with salinity of 22 ppt, temperature of 28 ± 1 °C, photoperiod of 12h: 12h (light: dark), conductivity of 750 ± 50 μS/cm, pH 7.5 ± 0.5 and dissolved oxygen equal to or greater than 95% saturation (these conditions were also used for the toxicity assays). Partial exchanges of the water (70-80%) were performed daily. The larvae were fed daily with newly hatched Artemia nauplii *ad libitum*.

Toxicity assays were performed using 2 days old larvae exposed for 4 days to 0.00, 0.02, 0.03, 0.04, 0.06, 0.10 and 0.15 μg/L of cypermethrin. The test was deployed in 6-well polyethylene plates. Five replicates per treatment were used and each replicate consisted of 5 larvae in a well with 10 ml of test solution. The test solution was renewed daily after feeding the larvae with newly hatched Artemia. Mortality of larvae was checked daily using a stereoscopic microscope. At the end of the test larvae were anesthetized with carbonated water and fixed in 70% ethanol for further determination of developmental stage (zoea), carapace length and number of internal lipid droplets, using a stereoscopic microscope. Zoeae (Fig. S1) were identified using specialized literature (Fincham, 1979). Carapace length (CC) was measured along the distance between the tip to the median posterior margin of the carapace (Soares et al., 2017). The number of lipid droplets is a measure of the energy reserves of the larvae and can be observed in the hepatopancreas region of the cephalothorax, representing remaining fat droplets of the egg yolk (Anger and Hayd, 2010).

2.2.2. Normal locomotion patterns of *P. varians*

In order to assess Barrage® effects on the locomotor behavior of *P.* varians, normal locomotion patterns of non-exposed larvae had to be understood and protocols defined. Zebrabox tracking system (ZEB 478 Viewpoint) equipped with an infrared camera of 25 frames per second was used for video tracking the movement of larvae individually exposed in 24-wells plates. After preliminary tests (results not shown) two protocols were established.

In the first protocol an acclimation period of 5 min in the darkness was followed by 1 minute period in the light and 1 minute period in the light. During this time distance (mm) and time (seconds) larvae spent swimming was recorded. Distance and time swam in specific types of movements were also recorded: inactivity movements for velocities below 0.5 cm/sec, slow movements between 0.5 and 3.0 cm/sec and rapid movements for velocities above 3.0 cm/sec. Moreover, reading arena was divided in two areas, an outside area and an internal area, were movements was recorded independently so that changes in swimming patterns could be detected (swimming in the edges of the well vs. in the center of the well). Moreover, eight classes of path angles were defined (see Fig. 3B) and the number of angles in each category was recorded. A transparent background and a detection threshold of 8 were used.

In the second protocol, *P. varians* larvae were subjected to an initial period of 5 min of darkness (acclimation) followed by 3 cycles of 0.33 min (20 secs) of light and 1 min of darkness to assess persistence of the response through cycles. These protocols were used to assess normal locomotor activity in 24 larvae (six days old).

2.2.3 Shrimp locomotion assay

The assay for assessment of Barrage® effects in the locomotor behavior of *P. varians* larvae was conducted with adjusted concentrations (0, 0.001, 0.003, 0.01, 0.03 μg/L of cypermethrin) where no mortality was observed. This test followed the same procedure as previously described in section 2.1.1 except that 24-well plates were used for exposure. One larva was placed per well with 2 ml of test solution and a total of 36 embryos per concentration were used. At the end of the test, movement of larvae was evaluated using the procedures described in section 2.2.2.

2.3 Zebrafish locomotion assay

D. rerio (AB wild type) embryos were supplied by the zebrafish facility at the Biology Department of the University of Aveiro, Portugal. Adult organisms were kept under controlled conditions, in a ZebTEC recirculation system (Tecniplast). The culture water used was tap water filtered by activated charcoal and reverse osmosis, complemented with "Instant Ocean Synthetic Sea Salt" (Spectrum Brands, USA) and automatically adjusted for pH and conductivity. The water temperature was 27.0 ± 1° C, conductivity 794 ± 50 μS / cm, pH 7.5 ± 0.5 and dissolved oxygen equal to or greater than 95% saturation. A photoperiod cycle was maintained 12h: 12h (light: dark). Adult fish were fed once daily with commercially available artificial diet (ZM-500 fish food; Zebrafish Management Ltd, UK). Zebrafish embryos were obtained as described in Andrade (2017) and exposed to sub-lethal concentrations of cypermethrin (0.0, 10.0, 25.0, 62.5, 156.0 and 390.0 μg/L) in 24-well plates according to OECD guideline 236

(OECD, 2013) during 5 days. Concentrations were selected based on previous work (Soares et al., 2017) and do not cause mortality in zebrafish larvae. Tests were performed under the same conditions as described for the culture using 16 embryos per concentration placed individually in the wells of a 24-well plate with 2 ml of test solution. The eggs were distributed in the plate using a randomized design. In the video tracking system Zebrabox larvae were subjected to light for 3 min, followed by a period of 3 min of darkness. Detection threshold was set to 20, inactivity movements were defined by velocities below 0.5 cm/sec, slow movements between 0.5 and 10.0 cm/sec and rapid movements for velocities above 10.0 cm/sec. Parameters measured were as described for shrimp, including classes of angles. Additionally, larvae were subjected to a second protocol characterized by cycles of 2 min light and 1 min of darkness (until a total time of 15 min) to assess persistence of the response through cycles. In this protocol a detection threshold of 16 was used.

2.4 Statistical analysis

Statistical analysis was performed using SigmaPlot software (version 12.5, Systat Software Inc., CA, USA) (Systat Software, 2014). All data were tested for normality (Shapiro-Wilk) and homoscedasticity. LC50 values were calculated by adjusting the data to a five parameters log-logistic function. A $\chi 2$ test was used to assess effects of cypermethrin on the developmental stages (zoea) of shrimp.

Generally, data was submitted to One-way analysis of variance (ANOVA) to assess effects of Barrage® treatments followed by the Dunnett's test to discriminated differences towards control, or in the case of data not normally distributed to a Kruskal-Wallis test followed by the Dunn's or Holm Sidak post-hoc test. Special data sets such as times and distances of shrimp larvae measured along a monitoring time gradient were subjected to a One-way Repeated Measures (RM) ANOVA or to a Friedman Repeated Measures ANOVA on Ranks (For non-normally distributed data) followed by a Dunnett's test. Data set from the repeated stimulus experiment was subjected to a Two-way RM ANOVA with Barrage® concentrations and responses in the light (for shrimp) or in the dark (for zebrafish) as factors. All analysis were performed with a level of significance of 0.05.

3. Results

3.1 Acute test with shrimp larvae

Cypermethrin, through the Barrage® formulation, caused significant effects on survival, carapace length, amount of energy reserves and development in *P. varians* larvae (Fig. 1). LC_{50} values calculated varied from 0.06 µg/L at 48 hours of exposure to 0.04 µg/L at 96 hours (Table 1 and Fig. 1A).

Table 1 – LC_{50} values, and respective standard error for larvae of *P. varians* Cypermethrin was used as Barrage® commercial formulation.

Time (h)	LC_{50}(µg/L)	Standard error	Model
48	0.065	0.932	LL.5
72	0.051	0.896	LL.5
96	0.045	0.916	LL.5

LL.5 - curve fit model = log-logistic five parameters function

Effects of the compound on larval development were clearly observed (Fig. 1B, Chi-square= 55.31, p<0.001). At the end of the test, the control larvae of *P. varians* were at zoea 5, while at the cypermethrin concentration 0.06 µg/L the larvae remained predominantly at zoea 4. Larval growth was also affected with all concentrations of cypermethrin tested eliciting a significant reduction in the carapace size of the *P. varians* larvae in relation to control (Fig. 1C; One-way ANOVA, F=42.92, p<0.001). The number of lipid droplets was also drastically reduced by cypermethrin (Fig. 1D; Kruskal-Wallis, H = 59.25, p <0.001).

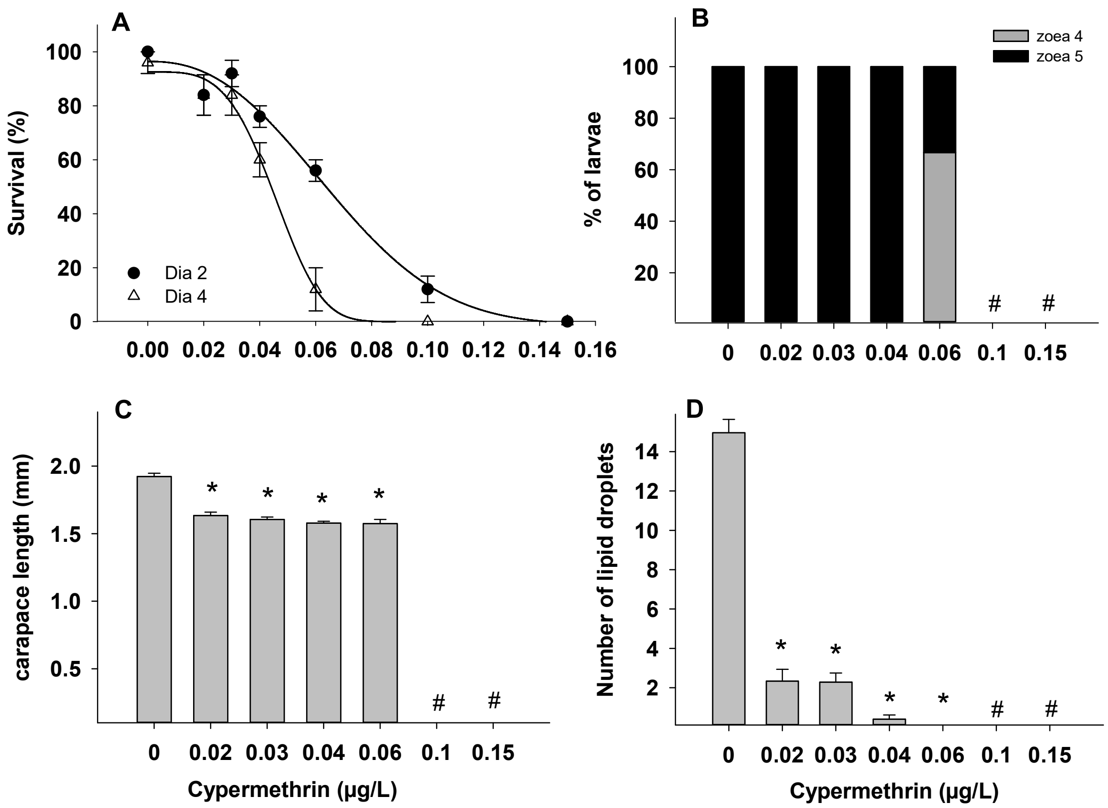

Figure 1. Effects of cypermethrin on *P. varians* shrimp larvae after 4 days of exposure: survival (A); stage of development (B) carapace length (C) and amount of lipid droplets (D). The asterisks showed statistically significant differences in relation to the control (Dunn's or Dunnett's test). "#" indicates insufficient survival no analyze the parameter. Values represent averages and error bars represent standard errors.

3.2 Basal locomotor behavior in *P. varians* larvae

The sudden change in lighting conditions (dark to light) causes a response in *P. varians* larvae characterized by an increase in activity in periods of light (supplementary material Fig. S2 and Fig. 2). Generally, in the dark, larvae present very low locomotor activity and after a sudden transition to light, larvae presented a short (20 seconds) peak of activity which immediately decreased and eventually returns to the initial state. This is the case of the swimming distance (One-way RM ANOVA, $F= 13.52$, $p< 0.001$, Fig. 2A), the distance traveled in rapid movements (One-way RM ANOVA, $F= 8.62$, $p< 0.001$, Fig. 2C) and the time traveled in rapid movements (One-way RM ANOVA, $F= 7.54$, $p< 0.001$, Fig. 2G). The % of distance and time traveled in the outside area of the arena responded in a different pattern slowly increasing after the switch. This was significant for % time (One-way RM ANOVA, $F= 2.38$, $p= 0.039$, Fig. 2F) but not for % distance (One-way RM ANOVA, $F= 2.02$, $p= 0.076$, Fig. 2B). Swimming time and distance and time swam in slow movements were not affected by changes in lighting conditions (Fig. 2D, E and H).

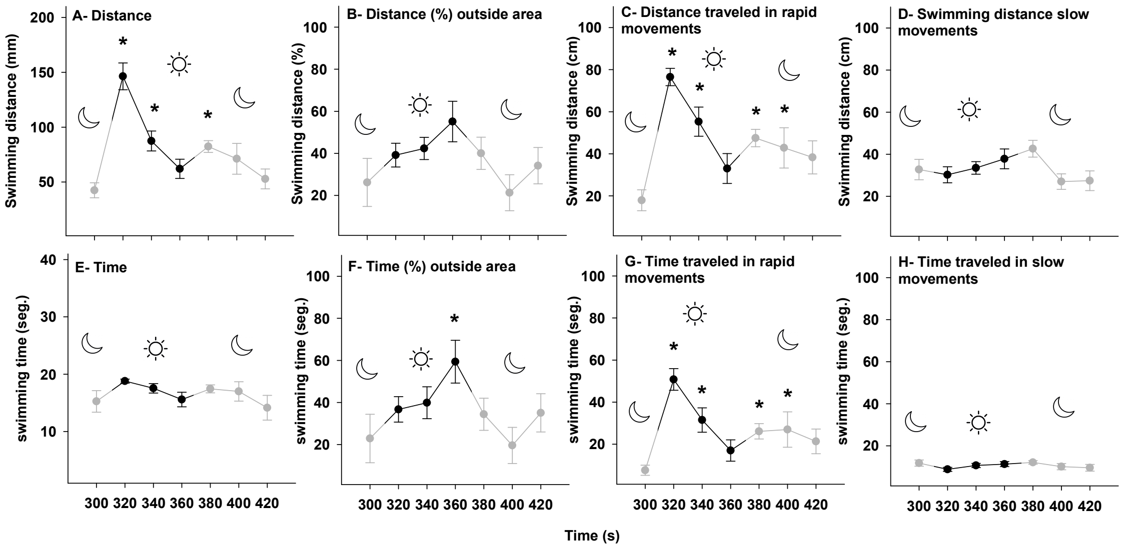

Figure 2. Locomotor response of larvae of *P. varians*. Effects (mean values ± standard error) of light and dark exposure: Swimming distance (A), distance traveled in the outside area (B), distance traveled in rapid movements (C), distance traveled in slow movements (D) swimming time (E), time swam in the outside area (F), time traveled in rapid movements (G) and time traveled in slow movements (H). The sun and the black dots represent periods of light and the moon and the grey dots represent dark periods. The asterisks showed statistically significant differences in relation to the first dark period (300 s) ($p < 0.05$, Holm-Sidak test).

The sudden change in lighting conditions (dark to light) also causes a response in the path angle of the larvae of *P. varians* characterized by a decrease in the frequency of class 5 angles after the switch to light followed by a slow return to original levels (Fig. 3; One-way RM ANOVA, $F = 2.72$, $p = 0.02$). The other swimming classes were not affected by the change in lighting conditions.

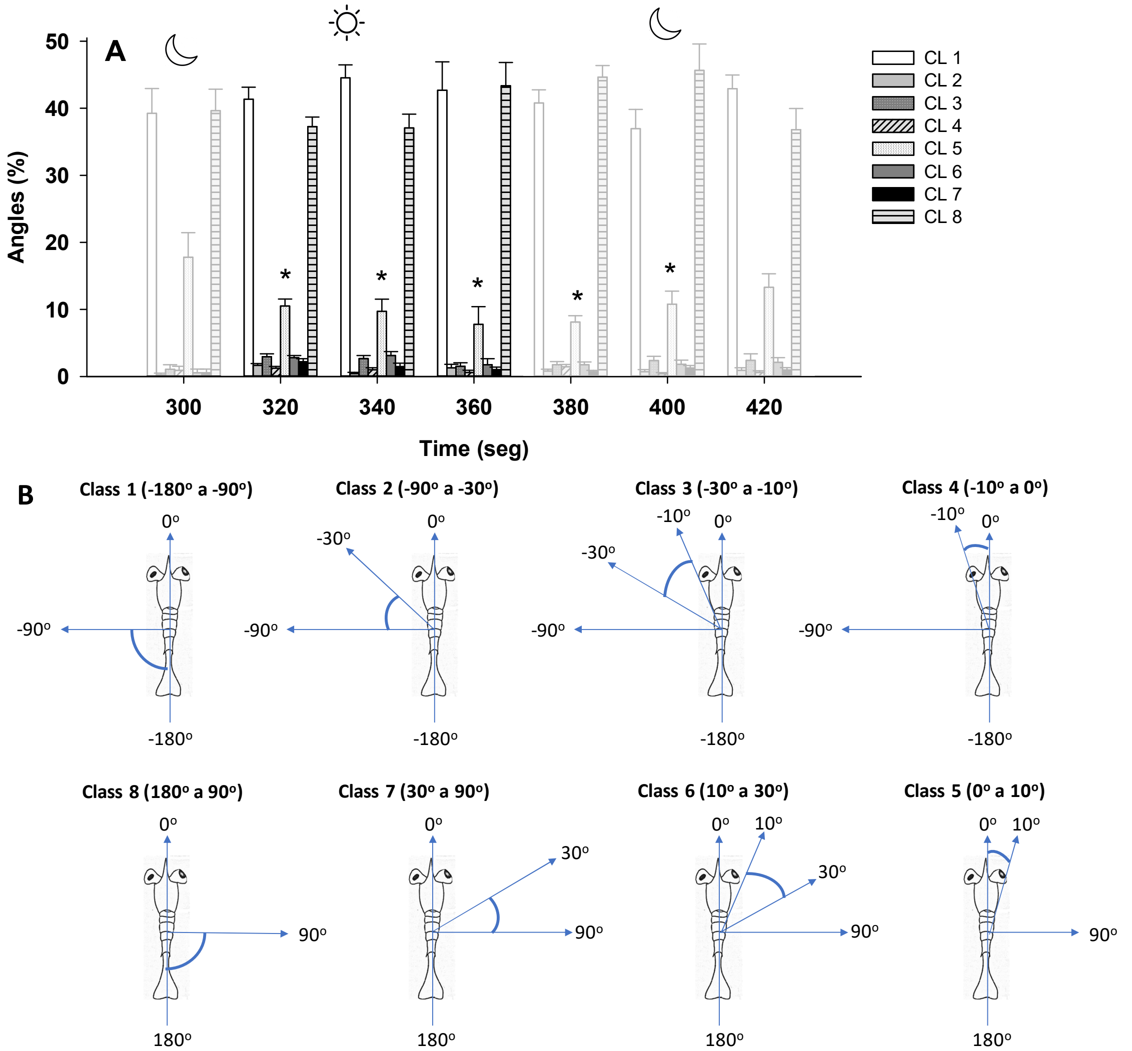

Figure 3. Effects of light and dark on the eight angular classes of the swimming path of larvae of *P. varians* (A) and scheme of the swimming classes measured (B). Values represent averages and error bars represent standard errors. The sun represents periods of light and the moon and the faded bars represent dark periods. The asterisks showed statistically significant differences in relation to the first dark period (300 s) (p <0.05, Holm-Sidak test).

The repeated switch from dark to light results in a decrease in the intensity of response to the stimulus as can be seen in Fig 4. This can be clearly observed in the distance moved (One-way RM ANOVA, F = 19.77, p = <0.001, Fig. 4A): while in the first switch the distance swam increased from 400 to 1200 mm, in the third switch activity increased from 400 to ~600. This effect was not observed for swimming time (Friedman test, $X^2 = 6.261$, p = 0.04, Fig. 4B).

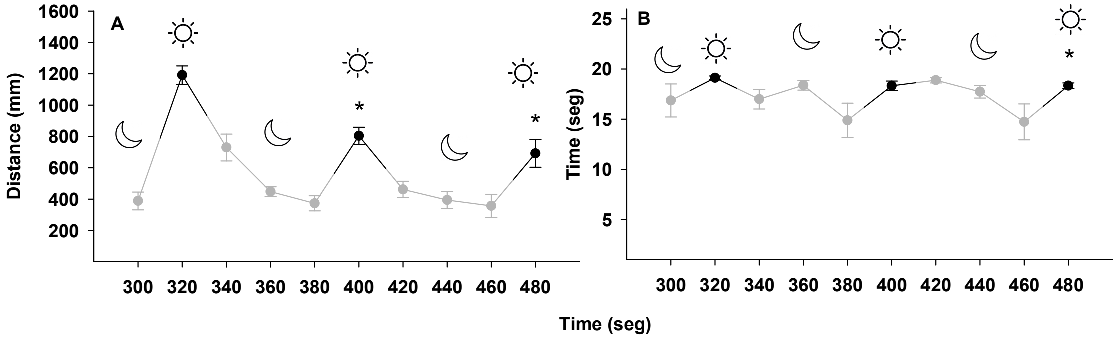

Figure 4. Locomotor response of shrimp larvae *P. varians*: swimming distance (A) and swimming time (B). Values represent averages and error bars represent standard errors. The sun and the black dots represent periods of light and the moon and the grey dots represent dark periods. The asterisks showed statistically significant differences in relation to the first dark period (300 s) for distance (Holm-Sidak test, p= <0.001) and for time (Dunnett's test q'= 3.46, p <0.05).

3.3 Behavioral effects of Barrage® in *P. varians* larvae

Effects of cypermethrin (via the Barrage® formulation) in the locomotion of shrimp larvae are depicted in Fig. 5 and 6. The locomotion levels in the dark and the response to the sudden switch from dark to light were not affected by Barrage® exposure (Fig. 5 and 6 periods A and B) except for swimming time after the switch which was not so increased at concentrations of 0.01 and 0.03 µg/L (Fig 5, A2; Kruskal Wallis, H= 14.18, p= 0.007). In the analysis of the types of movements (slow versus rapid) a clear effect can however be observed: exposed larvae responded to the stimulus with similar intensity as the control (increased rapid movements (Fig. 5 and 6, C2) and decrease of slow movements (Fig. 5 and 6, D2)), but while control organism returned to anterior levels during the following periods (periods 3 and 4), exposed larvae recovery was slower or incomplete during the monitoring time. This was however only partly supported by statistics with significant effects in the distance travelled in rapid movements, period 4 (Fig 6. C4, One-way Anova, F= 2.789, p= 0.036) and in the distance travelled in slow movements, period 4 (Fig. 6 D4, One-way ANOVA, F= 2.789, p= 0.036).

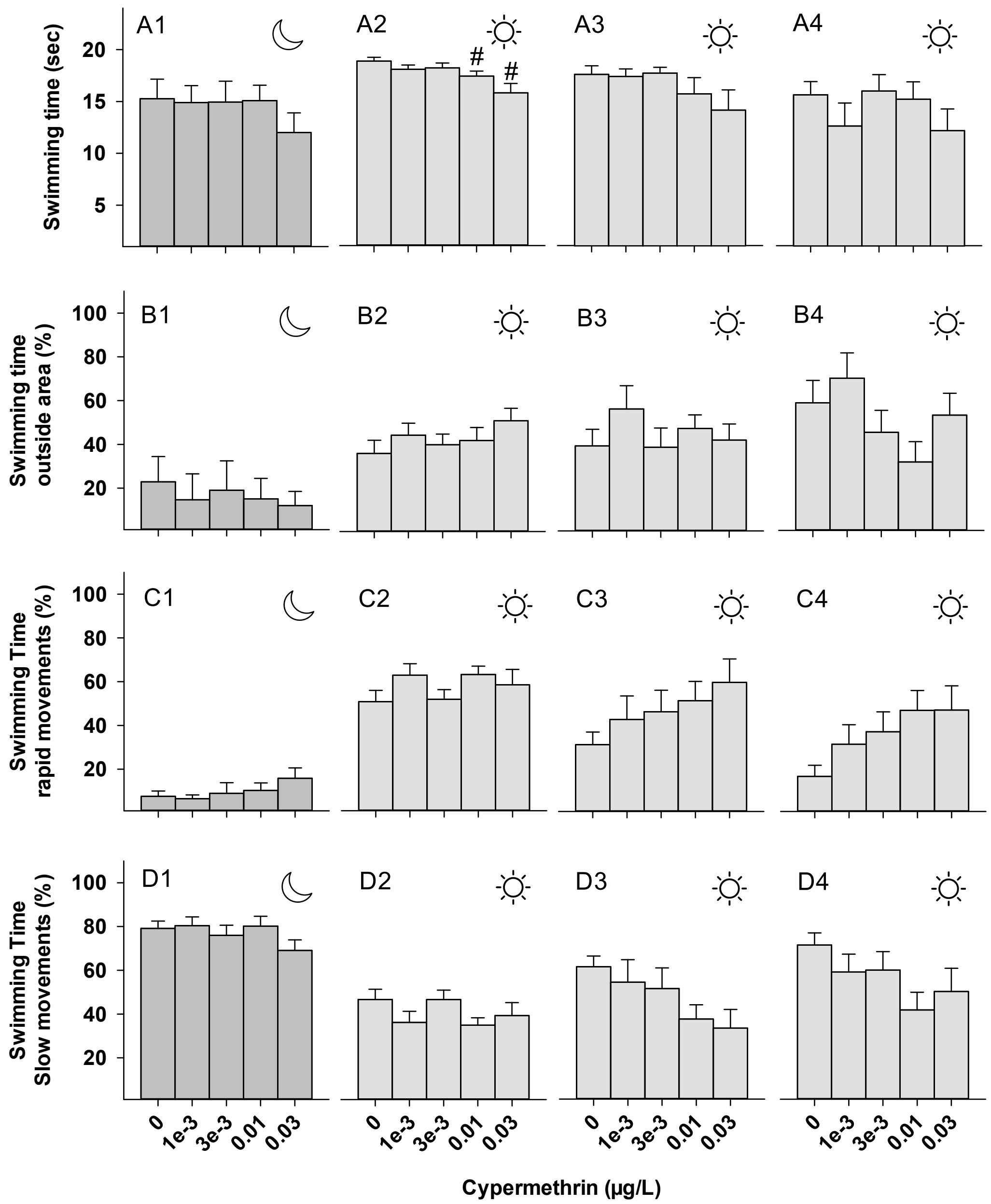

Figure 5. Effects of cypermethrin on the locomotor response (time) of *P. varians* larvae in 4 periods: 1 (after acclimation in the dark), 2 (20 seconds after the dark/light switch), 3 (40 seconds after the switch) and 4 (60 seconds after the switch). Values represent averages and error bars represent standard errors for swimming time (A); swimming time in the outside area (B); time swam in rapid movements (C) and time swam in slow movements (D). The moon and the dark grey bars represent periods of darkness and the sun and the light grey bars represent light periods. The # show statistically significant differences in relation to the dark period ($p < 0.05$, Holm-Sidak test).

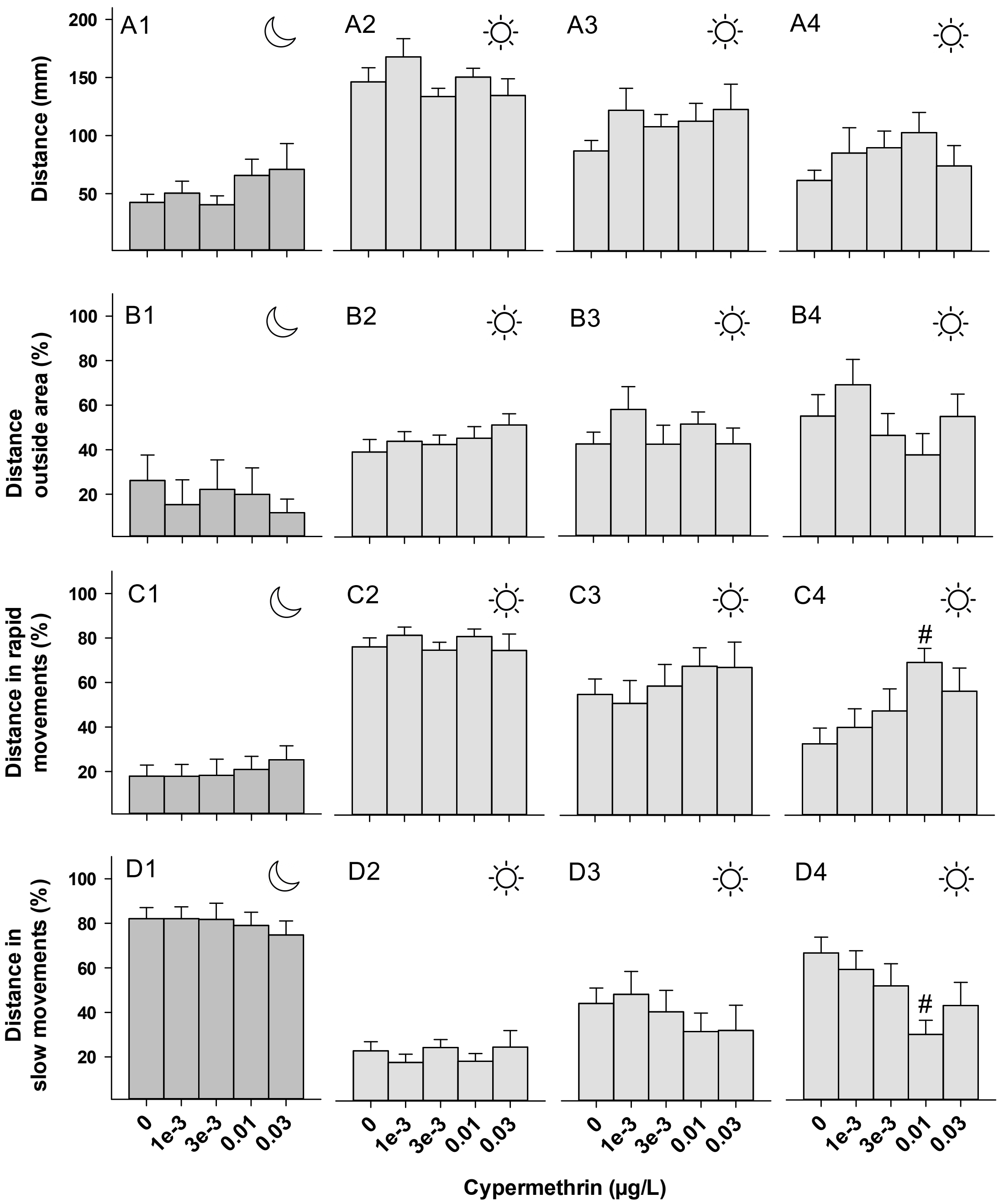

Figure 6. Effects of cypermethrin on the locomotor response (distance) of *P. varians* larvae in 4 periods: 1 (after acclimation in the dark), 2 (20 seconds after the dark/light switch), 3 (40 seconds after the switch) and 4 (60 seconds after the switch). Values represent averages and error bars represent standard errors for swimming distance (A); swimming distance in the outside area (B); distance swam in rapid movements (C) and distance swam in slow movements (D). The moon and the dark grey bars represent periods of darkness and the sun and the light grey bars represent light periods. The # show statistically significant differences in relation to the dark period ($p < 0.05$, Holm-Sidak test).

No effect of cypermethrin was observed on the path angle of *P. varians* larvae exposed to Barrage® (Fig. S3).

Under a repetition of the stimulus (switch from light to dark) larvae decreased the intensity of the response as expected (Fig. S4). The exposure to Barrage® did not change this response pattern as proven by the absence of interaction in the Two-way RM ANOVA (time: F= 1026, p= 0.421 and distance: F= 1.036, p=0.414)

3.3 Effect of cypermethrin on the behavior of zebrafish

Exposure to Barrage® caused a dose dependent inhibition of locomotor activity of zebrafish larvae in all parameters tested in both dark and light periods (Fig. 7 and Table S1).

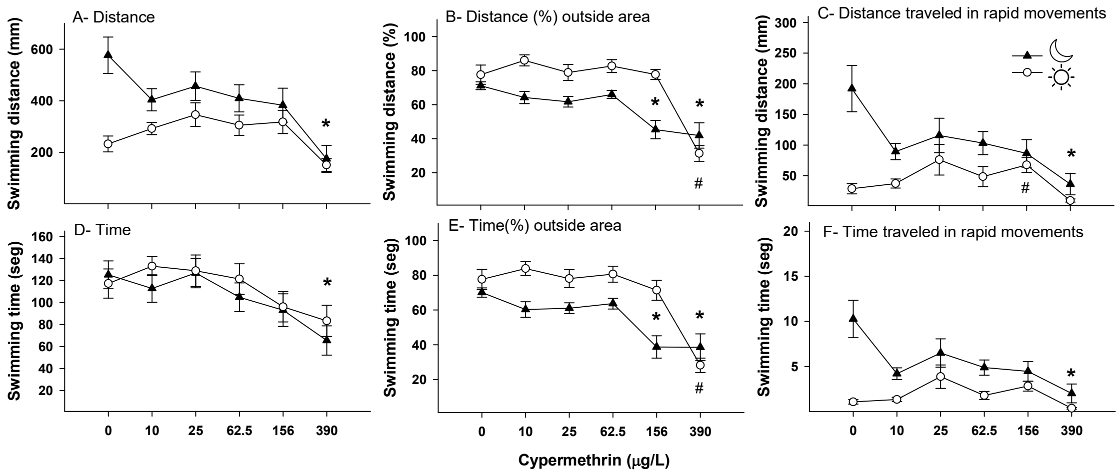

Fig 7. Effects of cypermethrin on the locomotor response of zebrafish embryos: swimming distance (A); distance swam in the outside area (B); distance swam traveled in rapid movements (C); swimming time (D); time swam in the outside area (E) and time traveled in rapid movements (F). White circles represent the light period and black triangles represent the dark period. Statistically significant differences regarding the control are indicated with a * for dark periods and # for light periods (Dunn's test or Dunnett's test; p <0.05).

The effects of Barrage® on the path angle of zebrafish larvae are shown in Fig. 8 and Table S1. The highest concentration of cypermethrin tested (390 µg/L) showed a significant effect in all classes of angles defined in both periods of light and dark with an increase in high amplitude angles (classes 1, 2, 7 and 8) and a decrease of low amplitude angles (classes 4 and 5).

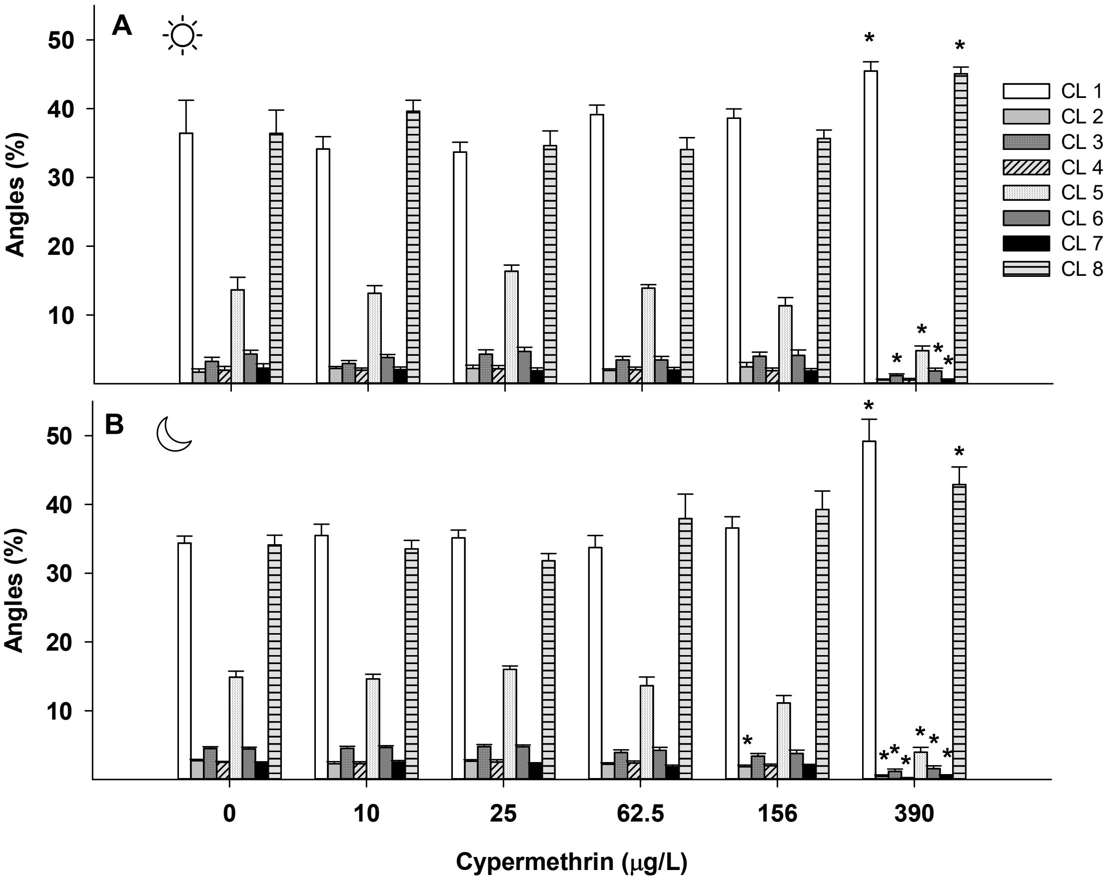

Figure 8. Effect of Barrage® on path angles of zebrafish larvae: angular classes in light (A) and dark (B). Values represent averages and error bars represent standard errors. The sun represents the period of light and the moon represents the dark period. The asterisks showed statistically significant differences in relation to the control for cypermethrin (Dunn's test or Dunnett's test, $p < 0.05$).

Zebrafish larvae seems to respond with the same intensity to repeated stimulus (light/dark switch) (Fig. S5). Barrage® exposure did not seem to alter this response pattern as indicated by the absence of interaction between the stimulus number and Barrage® concentrations in the Two-way RM ANOVA (time: $F= 1041$, $p= 0.412$ and distance: $F= 1.336$, $p=0.152$).

4. Discussion

This work aimed to develop methodologies to assess locomotor behavior in larvae of the shrimp *P. varians* using the automated video tracking system Zebrabox. In addition, the acute effects of the cypermethrin based formulation Barrage® to larvae of *P. varians* and behavioral evaluation in comparison to *D. rerio* were assessed.

Cypermethrin, through the Barrage® formulation, was found to be toxic to *P. varians* larvae, affecting its survival, development and increased energy reserve consumption. A 96h-LC_{50} value of 0.04 μg/L was calculated which is similar to the obtained for larvae of *Macrobrachium pantanalense* (0.05 μg/L) and *M. amazonicum* (0.10 μg/L) reported in a previous study of the group for the same cypermethrin-based formulation (Soares et al., 2017). The effects of Barrage® on body length, larvae development and consumption of energy reserves previously observed for *M. pantanalense and M. amazonicum* (Soares et al., 2017) were also confirmed in this work for *P. varians.* Reduction of body length and lipid droplets were observed from the lowest concentration tested (0.02 μg/L) reinforcing the great sensibility of shrimp larval stages to this compound. Growth reduction was also observed in *Palaemonetes argentinus* shrimp exposed to concentrations as low as 0.0001 μg/L of cypermethrin (Collins and Cappello, 2006). Increased consumption of reserves is the result of the increase in the metabolism to fight the aggression to the organism posed by the toxic compound and leads to a shortage of reserves available for growth and development (Hayd et al., 2014).

Locomotor responses of *P. varians* larvae were analyzed to understand the potential of the species as a model for behavioral ecotoxicology. A sudden change in lighting conditions may constitute a stress event to organisms. In the case of zebrafish larvae, hyperactivity is observed when switching from light to dark (Henriques et al., 2016). Unlike zebrafish, *P. varians* larvae presented a burst of activity (hyperactivity) upon the switch from dark to light (startle response). This can be observed especially measuring total distance swam and the distance travelled in rapid movements. Swimming pattern also differs with larvae swimming predominantly in the edges of the well in detriment of the center (increased time swimming in the outside area). This hyperactivity however does not last a long time with larvae returning to basal levels within 1 minute. The analysis of path angle revealed a decrease of class class 5 angles (low amplitude) and an increase of class 1 angles (high amplitude) suggesting a more erratic pattern of movement in the light and supporting the hyperactivity observed through the measurement of the swimming distances. Shrimp larvae also showed the capacity of adaptation to the stress factor by decreasing the intensity of the response in subsequent starles. Colón-Cruz et al., (2018) also evaluated the locomotor response using ViewPoint's Zebrabox with a protocol of five intercalated periods of light / dark cycles lasting 10 minutes for larvae of *Macrobrachium carcinus* and greater activity was observed during light cycles, while *M. rosenbergii* was most active during dark cycles. We can observe that the behavioral response to stress can vary according to the species, even if they are very related. To try to explain the difference, the authors argue that the difference may be linked to the circadian rhythm and whether the animals are diurnal or nocturnal.

Both *P. varians* and *D. rerio* larvae showed alterations in locomotor behavior after exposure to Barrage®, however shrimp larvae presented higher sensitivity than zebrafish. Few studies have reported behavioral effects of pyrethroids in crustaceans. Melvin and Wilson (2013) compiled studies evaluating behavior, lethality (LC50), development and reproduction reporting that in crustaceans and fish, behavior is more sensitive than developmental and reproductive endpoints. In this study the lowest concentrations eliciting behavior effects were 0.01 μg/L for shrimp and 156 μg/L for zebrafish; approximately 4 and 10 times lower then respective LC_{50} 96h values (0.04 (present study) and 96h 1340 μg/L (Soares et al., 2019, in preparation).

Barrage® did not have an effect on the response of larvae to the startle but while control organisms returned to basal levels almost immediately, exposed larvae had a slow or incomplete return to normal levels. This means that exposure to the pesticide interfered with the recovery capacity from the stress factor, confirming the neurotoxic effects of cypermethrin (Keith and Walker, 1992; NPTN, 1998) in *P. varians.* Lack of significance for some parameters where a tendency is clearly observed is due to data variability and should be overcome by increasing N in future studies. Collins and Cappello (2006) examined the effect of cypermethrin on the juveniles shrimp *Palaemonetes argentinus* at 0.025 μg/L, observing effects such as hyperactivity (shifted and circle swimming immediately after they were placed in the pyrethroid solution) and effects in the ecdysis (intermolt time was severely altered, increasing the period between seedlings and the rate of growth was reduced). These authors explain that cypermethrin being neurotoxic could affect the regulation of the synthesis process in nerve cells and in the transmembrane sodium influx, especially in the neuro-hormonal system. Already Christensen et al. (2005) examined the effects of cypermethrin on *Daphnia magna* after 6 hours exposure observing alterations on swimming ability (inhibition of swimming, tremors) and feed efficiency (feeding reduction) at concentrations of 0.1 μg/L. Oliveira et al. (2012) studied the effects of deltamethrin, a pyrethroid with cypermethrin-like action on the behavior of *Palaemon serratus*, observed a decrease in swimming velocity at concentrations from 0.6 ng/L.

Changes in the behavior of a species can compromise vital activities such as feeding and breathing, as well as the use of environmental resources and interaction with other organisms (Denoël et al., 2013). Organisms with altered swimming efficiency are more susceptible to predation, changing the predator-prey interaction with consequences for the equilibrium of aquatic communities (Henriques et al., 2016).

In this study *D. rerio* showed behavioral effects caused by Barrage® at concentrations from 156 µ/L. Barrage® caused a dose-dependent decrease in the total distance and in the distance and time travelled in rapid movements especially in the dark periods. Previous studies reported reduced swimming speed of zebrafish larvae exposed to 10 µg/L of cypermethrin, confirming the induction of neurotoxicity (Li et al., 2018). In addition, it was reported an increment of the exploratory activity of the fish *Jenynsia multidentata*, exposed to cypermethrin (0.04 and 0.4 µg/L). Authors observed that fish swam in the upper part of the aquarium which is considered as an escape act. Moreover, inhibition of acetylcholinesterase activity in the muscle was observed and associated with a decrease in the swimming behavior with the increase of concentrations (Bonansea et al., 2016).

In summary, cypermethrin had effects on the behavior of shrimp larvae at 0.01 µg/L cypermethrin, whereas for fish, effects at 156 µg/L were observed. In the case of the shrimp these results show great ecological relevance since responses are observed at concentrations plausible to be found in aquatic environments (e.g. 3.3 ng/L were found in Californian surface water (Werner and Young, 2018), 107 ng/L were found in greek waters (Stehle and Schulz, 2015) and 13 ng/L were found in efluents of a sewage treatment plant in California (Markle et al., 2014).

This study highlights the importance of transferring methodologies to behavioral assessments for other species including crustaceans. For a better understanding of the ecological risk of Barrage® we suggest further studies with representative species of different levels in the food chain.

5. Conclusion

Methodologies for behavioral assessment using the video tracking system Zebrabox were successfully developed for *P. varians* larvae showing the potential of the species for behavior analysis. A sudden switch in light conditions (dark to light) constitutes a stimulus (startle) that elicits an increased motor activity characterized by an increment in the distance traveled, in the time spent along the edges of the wells and a decrease of low amplitude angles suggesting a pattern of hyperactivity and erratic swimming. However, the intensity of this response decreases with the repetition of the stimulus suggesting the adaptation of shrimp larvae to the stimulus.

Exposure of shrimp larvae to Barrage® elicited developmental effects similar to the observed for other shrimp species (growth inhibition, decrease of lipid droplets and developmental delay) suggesting energy allocation to fight Barrage® toxicity. At behavioral level, larvae exposed to Barrage® showed a slower or incomplete recovery from the response to the startle when comparing to the control. This was observed at concentrations as low as 0.01 µg/L, confirming the high sensitivity of behavioral parameters and rising concerns on the risk of Barrage® residues in the aquatic environment for shrimp species. Zebrafish behavioral response to Barrage® was observed at concentrations from 156 µg/L and were characterized by hypoactivity and erratic swimming.

This study reinforces the importance of behavior as an endpoint in risk assessment of chemical compounds and shows, through the Barrage® case-study, the potential of shrimp species as model organisms in behavior ecotoxicology.

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6. References

Andrade, T.S., Henriques, J.F., Almeida, A.R., Soares, A.M.V.M., Scholz, S., Domingues, I., 2017. Zebrafish embryo tolerance to environmental stress factors—Concentration–dose response analysis of oxygen limitation, pH, and UV-light irradiation. Environ. Toxicol. Chem. 36. https://doi.org/10.1002/etc.3579

Anger, K., 2001. The Biology of Decapod Crustacean Larvae, 14th ed. Lisse: AA Balkema Publishers.

Anger, K., Hayd, L., 2010. Feeding and growth in early larval shrimp *Macrobrachium amazonicum* from the Pantanal, southwestern Brazil. Aquat. Biol. 9, 251–261. https://doi.org/10.3354/ab00259

Barros, A. de, 1992. Recomendações para controle da mosca-dos-chifres no Pantanal. Embrapa, Centro de Pesquisa Agropecuária do Pantanal (Corumba, MS). [WWW Document]. ainfo.cnptia.embrapa.br.

Bonansea, R.I., Wunderlin, D.A., Amé, M.V., 2016. Behavioral swimming effects and acetylcholinesterase activity changes in *Jenynsia multidentata* exposed to chlorpyrifos and cypermethrin individually and in mixtures. Ecotoxicol. Environ. Saf. 129, 311–319. https://doi.org/10.1016/j.ecoenv.2016.03.043

Champagne, D.L., Hoefnagels, C.C.M., de Kloet, R.E., Richardson, M.K., 2010. Translating rodent behavioral repertoire to zebrafish (*Danio rerio*): Relevance for stress research. Behav. Brain Res. 214, 332–342. https://doi.org/http://doi.org/10.1016/j.bbr.2010.06.001

Christensen, B.T., Lauridsen, T.L., Ravn, H.W., Bayley, M., 2005. A comparison of feeding efficiency and swimming ability of *Daphnia magna* exposed to cypermethrin. Aquat. Toxicol. 73, 210–220. https://doi.org/10.1016/J.AQUATOX.2005.03.011

Collins, P., Cappello, S., 2006. Cypermethrin Toxicity to Aquatic Life: Bioassays for the Freshwater Prawn *Palaemonetes argentinus*. Arch. Environ. Contam. Toxicol. 51, 79–85. https://doi.org/10.1007/s00244-005-0072-1

Colón-Cruz, L., Kristofco, L., Crooke-Rosado, J., Acevedo, A., Torrado, A., Brooks, B.W., Sosa, M.A., Behra, M., 2018. Alterations of larval photo-dependent swimming responses (PDR): New endpoints for rapid and diagnostic screening of aquatic contamination. Ecotoxicol. Environ. Saf. 147, 670–680.

Correia, M., Palma, J., Andrade, J.P., 2016. Growth performance of the early life stages of broad-nosed pipefish, *Syngnathus typhle* (L.) fed different live or frozen diets. Aquac. Res. 47, 1652–1660. https://doi.org/10.1111/are.12635

Cottin, D., Brown, A., Oliphant, A., Mestre, N.C., Ravaux, J., Shillito, B., Thatje, S., 2012. Sustained hydrostatic pressure tolerance of the shallow water shrimp *Palaemonetes varians* at different temperatures: Insights into the colonisation of the deep sea. Comp. Biochem. Physiol. 162, 357–363.

https://doi.org/10.1016/j.cbpa.2012.04.005

Dell'Omo, G., 2002. Behavioural ecotoxicology. John Wiley & Sons.

Denoël, M., Libon, S., Kestemont, P., Brasseur, C., Focant, J.-F., De Pauw, E., 2013. Effects of a sublethal pesticide exposure on locomotor behavior: A video-tracking analysis in larval amphibians. Chemosphere 90, 945–951. https://doi.org/10.1016/j.chemosphere.2012.06.037

Fincham, A.A., 1979. Larval development of British prawns and shrimps (Crustacea, Decapoda, Natantia). 2. *Palaemonetes* (*Palaemonetes*) *varians* (Leach, 1814) and morphological variation. Bull. Br. Museum Nat. Hist. 35, 163–182. https://doi.org/10.3366/anh.1992.19.3.426

García-de la Parra, L.M., Bautista-Covarrubias, J.C., Rivera-de la Rosa, N., Betancourt-Lozano, M., Guilhermino, L., 2006. Effects of methamidophos on acetylcholinesterase activity, behavior, and feeding rate of the white shrimp (*Litopenaeus vannamei*). Ecotoxicol. Environ. Saf. 65, 372–380. https://doi.org/10.1016/j.ecoenv.2005.09.001

Geferson, A., Universidade, B., Helena, R., Mour, V., Federal, U., Biochemistry, A., Silva, L., 2015. Aquicultura no Brasil : Novas Perspectivas, ResearchGate. São Carlos, SP.

Gerhardt, A., 2007. Aquatic Behavioral Ecotoxicology—Prospects and Limitations. Hum. Ecol. Risk Assess. An Int. J. 13, 481–491. https://doi.org/10.1080/10807030701340839

Gomes, A., Koller, W., Barros, A., 2011. Susceptibility of *Rhipicephalus* (*Boophilus*) *microplus* to acaricides in Mato Grosso do Sul, Brazil. Ciência Rural 41, 1447–1452. https://doi.org/http://dx.doi.org/10.1590/S0103-84782011005000105

Gravato, C., Almeida, J.R., Silva, C., Oliveira, C., Soares, A.M.V.M., 2014. Using a multibiomarker approach and behavioural responses to assess the effects of anthracene in *Palaemon serratus*. Aquat. Toxicol. 149, 94–102. https://doi.org/10.1016/j.aquatox.2014.01.024

Harayashiki, C.A.Y., Reichelt-Brushett, A.J., Liu, L., Butcher, P., 2016. Behavioural and biochemical alterations in *Penaeus monodon* post-larvae diet-exposed to inorganic mercury. Chemosphere 164, 241–247. https://doi.org/10.1016/j.chemosphere.2016.08.085

Hart, J.L., Thacker, J.R., Braidwood, J.C., Fraser, N.R., Matthews, J.E., 1997. Novel cypermethrin formulation for the control of sea lice on salmon (*Salmo salar*). Vet.

Rec. 140, 179–81. https://doi.org/10.1136/VR.140.7.179

Hayd, L. a., Lemos, D., Valenti, W.C., 2014. Effects of Ambient Nitrite on Amazon River Prawn, *Macrobrachium amazonicum* , larvae. J. World Aquac. Soc. 45, 55–64. https://doi.org/10.1111/jwas.12071

Hellou, J., 2011. Behavioural ecotoxicology, an “early warning” signal to assess environmental quality. Environ. Sci. Pollut. Res. 18, 1–11. https://doi.org/10.1007/s11356-010-0367-2

Henriques, J.F., Almeida, A.R., Andrade, T., Koba, O., Golovko, O., Soares, A.M.V.M., Oliveira, M., Domingues, I., 2016. Effects of the lipid regulator drug gemfibrozil: A toxicological and behavioral perspective. Aquat. Toxicol. 170, 355–364. https://doi.org/10.1016/j.aquatox.2015.09.017

Keith, L.H., Walker, M., 1992. EPA’S Pesticide Fact Sheet Database. CRC Press.

Li, M., Wu, Q., Wang, Q., Xiang, D., Zhu, G., 2018. Effect of titanium dioxide nanoparticles on the bioavailability and neurotoxicity of cypermethrin in zebrafish larvae. Aquat. Toxicol. 199, 212–219. https://doi.org/10.1016/j.aquatox.2018.03.022

Luvizotto-Santos, R., Eler, M.N., Espindola, E.L.G., Vieira, E.M., 2009. O uso de praguicidas nas pisciculturas e pesqueiros situados na Bacia do rio Mogi-Guaçu. Bol. do Inst. Pesca 35, 343–358.

Markle, J.C., van Buuren, B.H., Moran, K., Barefoot, A.C., 2014. Pyrethroid pesticides in municipal wastewater: a baseline survey of publicly owned treatment works facilities in California in 2013. Descr. Behav. Eff. Pestic. urban Agric. settings 1168, 177–194.

Martins, M.L., 2004. Cuidados Básicos e Alternativas no Tratamento de Enfermidades de Peixes na Aqüicultura Brasileira, in: Editora Varela (Ed.), Sanidade de Organismos Aquáticos. pp. 355–368.

Maximino, C., de Brito, T.M., da Silva Batista, A.W., Herculano, A.M., Morato, S., Gouveia, A.J., 2010. Measuring anxiety in zebrafish: a critical review. Behav. Brain Res. 214, 157–171. https://doi.org/10.1016/j.bbr.2010.05.031

Melvin, S.D., Wilson, S.P., 2013. The utility of behavioral studies for aquatic toxicology testing: A meta-analysis. Chemosphere 93, 2217–2223. https://doi.org/10.1016/j.chemosphere.2013.07.036

Montanha, FP, C.P., 2012. Efeitos toxicológicos de piretróides (cipermetrina e deltametrina) em peixes-Revisão. Rev. Científica Eletrônica Med. Veterinária 18, 1–58.

New, P., Brown, A., Oliphant, A., Burchell, P., Smith, A., Thatje, S., 2014. The effects of temperature and pressure acclimation on the temperature and pressure tolerance of the shallow-water shrimp *Palaemonetes varians*. Mar. Biol. 161, 697–709. https://doi.org/10.1007/s00227-013-2371-9

NPTN, 1998. National Pesticide Information Center -Cypermethrin. Oregon.

OECD, 2013. Test No. 236: Fish Embryo Acute Toxicity (FET) Test, OECD Guidelines for the Testing of Chemicals, Section 2. OECD Publ. 1–22. https://doi.org/doi:10.1787/9789264203709-en

Oliveira, C., Almeida, J., Guilhermino, L., Soares, A.M.V.M., Gravato, C., 2012. Acute effects of deltamethrin on swimming velocity and biomarkers of the common prawn *Palaemon serratus*. Aquat. Toxicol. 124–125, 209–216. https://doi.org/10.1016/j.aquatox.2012.08.010

Palma, J., Bureau, D.P., Andrade, J.P., 2008. Effects of binder type and binder addition on the growth of juvenile *Palaemonetes varians* and *Palaemon elegans* (Crustacea: Palaemonidae). Aquac. Int. 16, 427–436. https://doi.org/10.1007/s10499-007-9155-5

Pavlaki, M.D., Araújo, M.J., Cardoso, D.N., Silva, A.R.R.S., Cruz, A., Mendo, S., Soares, M.V.M., Calado, R., Loureiro, S., 2016. Ecotoxicity and genotoxicity of cadmium in different marine trophic levels. Environ. Pollut. 215, 203–212. https://doi.org/10.1016/j.envpol.2016.05.010

Rainbow, P.S., Smith, B.D., 2013. Accumulation and detoxification of copper and zinc by the decapod crustacean *Palaemonetes varians* from diets of field-contaminated polychaetes *Nereis diversicolor*. J. Exp. Mar. Bio. Ecol. 449, 312–320. https://doi.org/10.1016/j.jembe.2013.09.022

Robinson, P.D., 2009. Behavioural toxicity of organic chemical contaminants in fish: application to ecological risk assessments (ERAs). Can. J. Fish. Aquat. Sci. 66, 1179–1188. https://doi.org/10.1139/F09-069

Silva, C., Oliveira, C., Gravato, C., Almeida, J.R., 2013. Behaviour and biomarkers as tools to assess the acute toxicity of benzo(a)pyrene in the common prawn *Palaemon serratus*. Mar. Environ. Res. 90, 39–46. https://doi.org/10.1016/j.marenvres.2013.05.010

Soares, M.P., Jesus, F., Almeida, A.R., Zlabek, V., Grabic, R., Domingues, I., Hayd, L., 2017b. Endemic shrimp *Macrobrachium pantanalense* as a test species to assess potential contamination by pesticides in Pantanal (Brazil). Chemosphere 168, 1082–1092. https://doi.org/10.1016/j.chemosphere.2016.10.100

Soares, M.P., Machado, A.L., Hayd, L., Soares, A., Domingues, I., 2019. Effects of pH and nitrites on the toxicity of a cypermethrin-based pesticide in zebrafish embryos. Prep.

Stehle, S., Schulz, R., 2015. Agricultural insecticides threaten surface waters at the global scale. Proc. Natl. Acad. Sci. 112, 5750–5755.

Systat Software, I., 2014. SigmaPlot for Windows Version 12.5 G, Germany.

Tu, H.T., Silvestre, F., Phuong, N.T., Kestemont, P., 2010. Effects of pesticides and antibiotics on penaeid shrimp with special emphases on behavioral and biomarker responses. Environ. Toxicol. Chem. 29, 929–938. https://doi.org/10.1002/etc.99

Werner, I., Young, T., 2018. Pyrethroid Insecticides—Exposure and Impacts in the Aquatic Environment. Encycl. Anthr. 5, 119–126. https://doi.org/https://doi.org/10.1016/B978-0-12-809665-9.09992-4

Supplemental Data

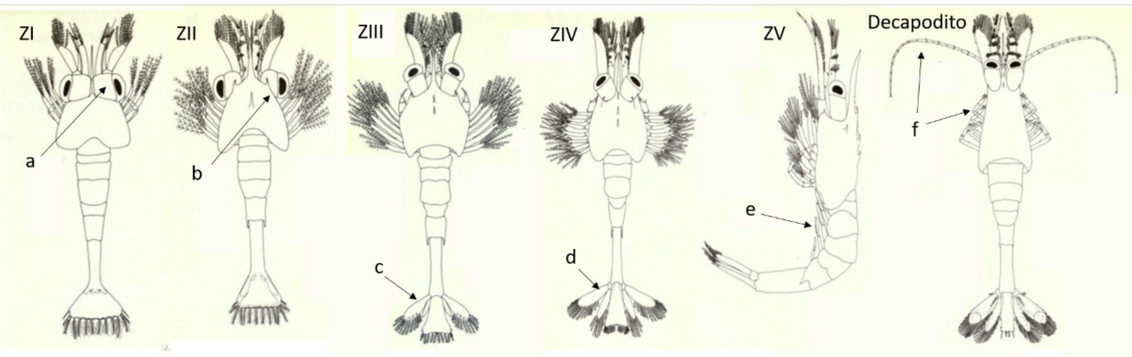

Figure S1- Structures for identification of the stages of larval development of *Palaemon varians*: ZI - Decapodito. (a) sessile eyes; (b) pedunculated eyes; (c) developed uropode endopodite; (d) developed uropopod endopodite and exopodite; (e) developed pleopods; (f) antenna and developed pereiopods. Source: Fincham (1979).

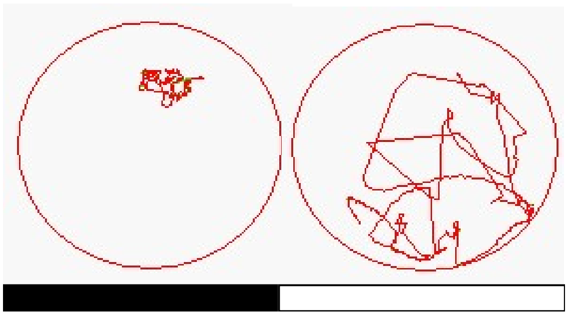

Figure S2- Representation of the swimming path of *Palaemon varians* larvae in a well of 24-wells microplates in periods of darkness (black bar) and light (white bar) (output of Zebrabox).

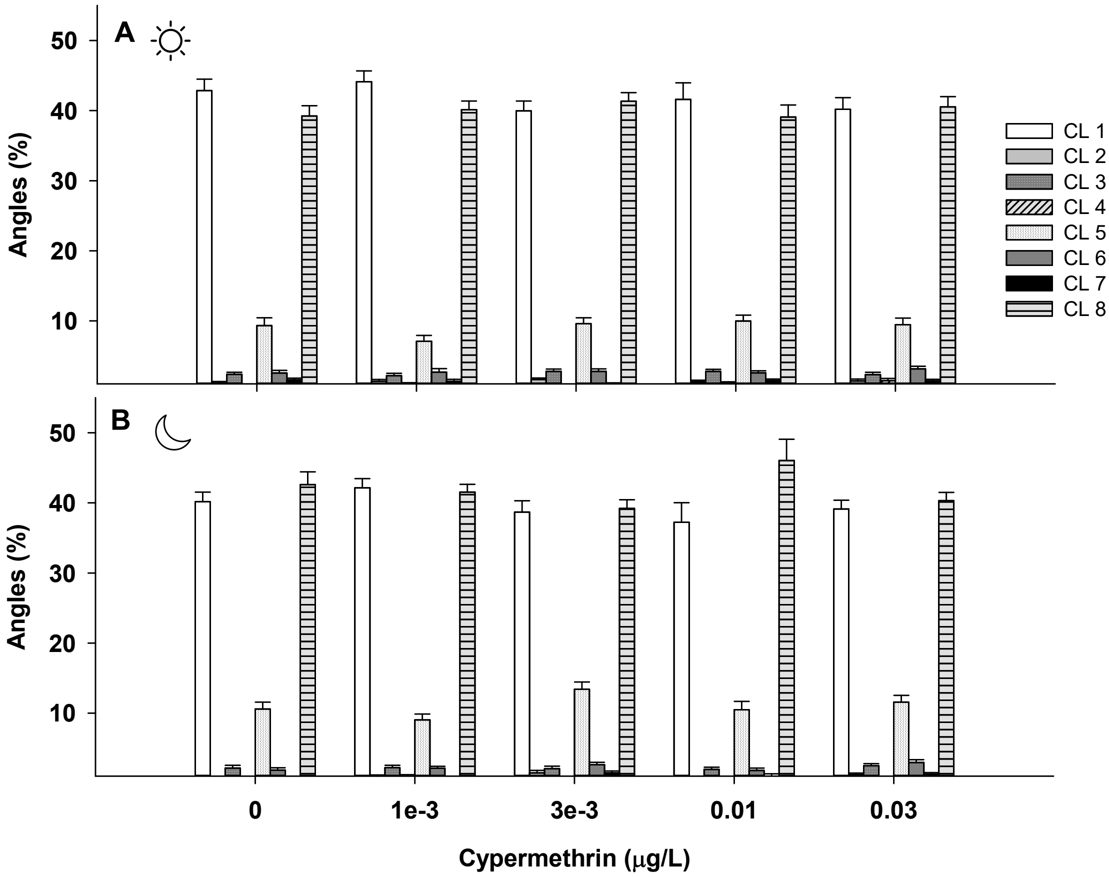

Figure S3- Effect of cypermethrin on angular swimming classes in larvae of *P. varians*: angular classes in light (A) and angular classes in dark (B). Values represent averages and error bars represent standard errors. The sun represents the period of light and the moon represents the dark period.

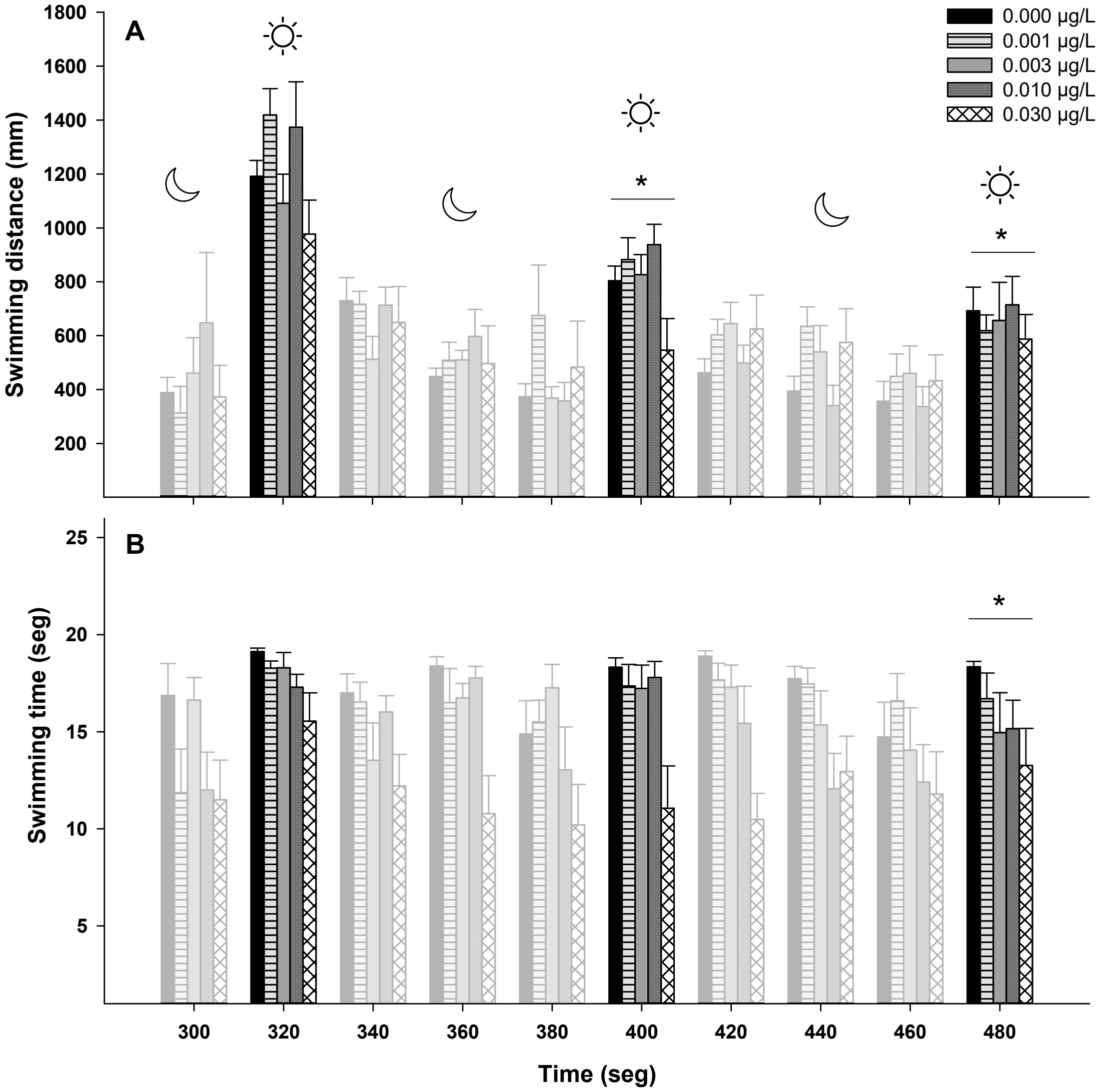

Figure S4. Effects of Barrage® on the response to repeated switches from dark to light in larvae of *P. varians*: Swimming distance (A) and swimming time (B). Values represent averages and error bars represent standard errors. The sun represents periods of light and the moon and faded bars represent dark periods. Asterisks show statistically significant differences towards the first light period (p <0.05, Holm-Sidak test).

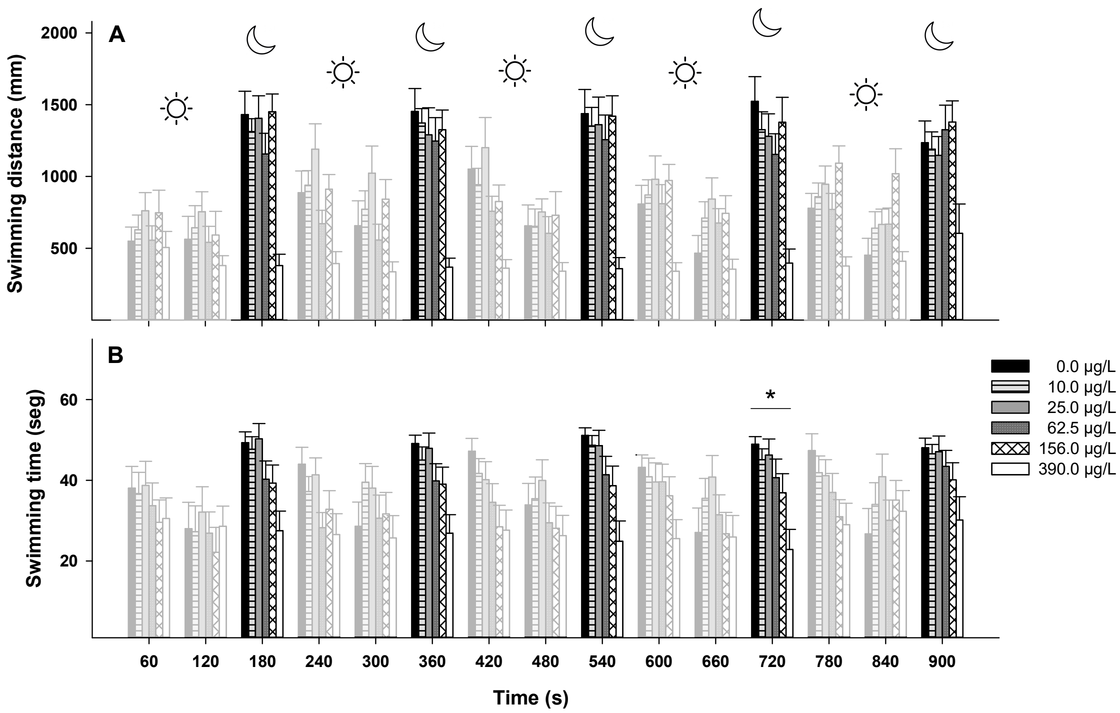

Figure S5. Effects of cypermethrin on adaptation to dark exposure in zebra fish embryos: Swimming distance (A) and swimming time (B). Values represent averages and error bars represent standard errors. The sun represents the period of light and the moon represents the dark period. Asterisks show statistically significant differences in relation to the time of first exposure of larvae to dark.

Table S1: Results of the statistical analysis performed on zebrafish locomotion data.

Parameter	Test	F or H value	P value
Swimming distance-light	One-way ANOVA	3.911	0.003
Swimming time-light	Kruskal-Wallis	11.63	0.040
Distance (% outside area)- light	One-way ANOVA	20.47	<0.001
Time (% outside area)- light	Kruskal-Wallis	30.	<0.001
Distance traveled in rapid movements-light	Kruskal-Wallis	17.91	0.003
Time traveled in rapid movements-light	Kruskal-Wallis	17.79	0.003
Swimming distance- dark	One-way ANOVA	5.30	<0.001
Swimming time- dark	Kruskal-Wallis	14.87	0.01
Distance (% outside area)- dark	Kruskal-Wallis	23.03	<0.001
Time (% outside area)- dark	Kruskal-Wallis	23.99	<0.001
Distance traveled in rapid movements-dark	Kruskal-Wallis	17.77	0.003
Time traveled in rapid movements	Kruskal-Wallis	19.57	0.002
Class 1- light	Kruskal-Wallis	27.49	<0.001
Class 2- light	Kruskal-Wallis	18.38	0.002
Class 3- light	Kruskal-Wallis	20.05	0.001
Class 4- light	Kruskal-Wallis	11.91	0.036
Class 5- light	Kruskal-Wallis	33.93	<0.001
Class 6- light	Kruskal-Wallis	15.30	0.009
Class 7- light	Kruskal-Wallis	13.06	0.023
Class 8- light	Kruskal-Wallis	22.33	<0.001
Class 1-dark	Kruskal-Wallis	30.22	<0.001
Class 2-dark	One-way ANOVA	11.92	<0.001
Class 3-dark	One-way ANOVA	14.92	<0.001
Class 4-dark	Kruskal-Wallis	32.12	<0.001
Class 5-dark	Kruskal-Wallis	41.31	<0.001
Class 6-dark	Kruskal-Wallis	25.98	<0.001
Class 7-dark	One-way ANOVA	10.80	<0.001
Class 8-dark	Kruskal-Wallis	25.37	<0.001

Chapter 6

General discussion

Discussion and concluding remarks

This thesis intended to contribute to the evaluation of effects of the cypermethrin-based insecticide Barrage® in aquatic species taking into account the variation of pH and nitrite concentrations. The species selected were the Pantanal endemic shrimp *Macrobrachium pantanalense*, the Amazon shrimp *Macrobrachium amazonicum* and the zebrafish (*Danio rerio*). Moreover, individual effects of Barrage® in very specific endpoints, histology and behavior, were studied using *M. pantanalense* and *Palaemons varians* repectively in order to understand more subtle effects of the compound in non-target organisms.

The pH levels tested showed direct effects on mortality and development of the two tested shrimp species and zebrafish embryos. In addition, pH modifies Barrage® toxicity at sublethal level for *M. pantanalense* (larval growth and development) and zebrafish embryos (development). Nitrite levels affected the growth of larvae of both shrimp species studied and also changed the effect of Barrage® on developmental levels in *M. amazonicum* and zebrafish. These results show the importance of considering abiotic factors, due to the possible direct effects on the physiology of organisms and interaction with existing contaminants. Furthermore, it suggests that the lethality may not be enough to correctly predict the combined effects of stressors due to the greater effects found at sublethal level (development). The importance of accounting for environmental variables and the use of endemic organisms and varying trophic levels in environmental impact assessments is demonstrated. The shrimp of Pantanal, *M. pantanalense* is of great relevance for the region, ecologically and for the local commerce. In the natural environments of the Pantanal this species plays a fundamental role in the food chain and as a bioindicator of environmental quality. Pantanal shrimp is also under pressure because it is widely used as a live bait for sport fishing, heavily exploited by tourism in the region (Catella 2003; Catella e Fernandes 2008; Karim et al. 2015). An accurate risk assessment of the pesticides used in the region for this shrimp species is thus crucial for its preservation. The Amazon shrimp, *M. amazonicum* also suffers anthropogenic pressure due to the degradation of estuarine environments in which this species relies for their larval development. Deforestation, accelerated growth of livestock and agriculture, fishing (artisanal fishing), sand dredging of canals and others, constitute risk factors for this species cooperating considerably to reduce its population in natural environments. Natural and global climate change-driven variation in environmental parameters adds to anthropogenic pressure rising concerns about the fitness of crustaceans species in a near future.

Behavior has been proven to provide a set of very sensitive tools that can be used as early warning signals in ecological risk assessment. The development of locomotor assessment in *P. palemons* larvae highlighted the potential of behavior to be used in larval stages of shrimp species using high performance systems as Zebrabox. Our data showed that the sudden change in light conditions (from dark to light) constitutes a startle to which larvae react by momentarily (20 seconds) increase speed. Larvae return to anterior activity levels rapidly (40 seconds). Barrage® exposure did not change the response to the startle but retarded the recovery. Our data showed a dose-response trend for this effect but statistically significance was only partly achieved. This calls the attention for the main drawback of the use of behavioral parameters which is data variability in these types of responses, highlighting the need of increase the N in behavioral analysis. Adaptation of protocols developed for *P. varians* to the Brazilian shrimp species would provide a important tool for environmental risk analysis of neurotoxic pesticides in Pantanal and Amazon basins.

Regarding the effects of Barrage® at histological level, our results showed important alterations in Pantanal shrimp gills, including relevant structural lesions that may affect the physiological function of these organisms. Gills constitute one of the main target organs of various chemicals present in the aquatic environment due to their direct contact with water and for this reason are considered an important organ for histological evaluation. Histopathological studies of other organs could however give complementary information on Barrage® mode of action and should be considered in future studies. Although effects were detected at relatively high cypermethrin concentrations they were obtained for a short time of exposure, calling the attention to the need of testing pesticides effects in ecological relevant scenarios such as long term exposures.

The results of this thesis clearly demonstrate that shrimp species are a very sensitive group that may suffer deleterious effects from the increasing use of pesticides in Pantanal. Data provided can raise awareness of competent entities, fomenting educational actions near the population for a sound and sustainable use of these compounds, systematic monitoring studies and risk evaluations and the implementation of mitigation measures and legislation for environmental protection so that ultimately this complex and important ecosystem can be preserved.

References

Catella, A.C., 2003. A pesca no Pantanal Sul: situação atual e perspectivas. Corumbá: Embrapa Pantanal (INFOTECA-E), 2003.

Catella, A.C., Fernandes, J., 2008. Estimativa da renda bruta dos pescadores de iscas vivas do Porto da Manga, Corumbá (MS) Embrapa Pantanal. Circular Técnica.

Karim, H.M., Freitas, J.E.C., Lima, T.P. de C., Nascimento, M.S., Hayd, L., 2015. Viabilidade econômica da produção do camarão-do-pantanal (*Macrobrachium pantanalense*). Bol. do Inst. Pesca 41, 103–112.